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*J Immunol* 2001; 166:6693-6703; doi: 10.4049/jimmunol.166.11.6693

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Flexibility in MHC and TCR Recognition: Degenerate Specificity at the T Cell Level in the Recognition of Promiscuous Th Epitopes Exhibiting No Primary Sequence Homology

Sunil K. Joshi, Padma R. Suresh, and Virander S. Chauhan

Recognition of peptide Ags by T cells through the TCR can be highly specific. In this report we show the degeneracy of Ag recognition at both MHC and TCR levels. We present evidence that unrelated promiscuous Th cell epitopes from various protein sources exhibit sufficient structural homology, despite minimal structural identity, to elicit cross-reactive proliferative responses at the bulk T cell level. This epitopic mimicry was also observed when peptide (CS.T3378–395 and TT830–844)-specific CD4+ T cell lines and T cell hybridoma clones were used in proliferation and Ag presentation assays. A scrambled CS.T3378–395 peptide did not show any proliferation, indicating that the specificity of the cross-reactive responses may be linked with the primary structure of the peptides. Blocking of CS.T3378–395-specific CD4+ T cell proliferation by anti-MHC class II mAb showed that recognition of promiscuous T cell epitopes is largely in association with MHC class II molecules. These findings suggest that promiscuous Th epitopes may be useful in designing peptide-based vaccine constructs. At the same time these results show that at the T cell level there may be a great deal of immunological cross-reactivity between heterologous pathogens, and because of this the host’s response to a pathogen may be modified by its previous experience with other unrelated pathogens.

Ne of the defining features of T cell activation is a high level of Ag specificity (reviewed in Ref. 1). However, several recent observations reveal that the TCR is highly flexible in its recognition, as it can recognize large number of different peptides that do not necessarily show strong sequence homology (2–12). It has become clear that a high level of degeneracy in TCR recognition is necessary to produce an adequate TCR repertoire capable of responding to the universe of pathogens as well as for the survival of T cells (reviewed in Ref. 13). The flexibility of the TCR recognition can manifest itself in a range of different biological outcomes depending on the affinity of MHC/peptide ligand to TCR, which includes antagonist and agonist effects. The structure of the MHC class II molecules allows the peptide to protrude and therefore accommodate longer peptides (15- to 23-aa residues) than MHC class I molecules that bind shorter peptides, mostly ranging from 9- to 12-aa residues. While the majority of antigenic peptides are thought to be recognized in the context of only one or a few MHC class II alleles, genetically promiscuous recognition in the context of several class II alleles has been reported in both murine and human systems (14–24). Although the molecular basis for peptide-related cross-reactivity as well as promiscuity is not yet clear, it is quite obvious that universal Th epitopes should have in their sequences structural features that allow them to interact with different MHC class II molecules and further, through the MHC/peptide complex, also enable them to interact with the TCR. As part of our interest in the use of universal Th epitopes in the design of peptide-based immunogens we have asked whether the peptide that can promiscuously bind various MHC class II molecules could also produce cross-reactive T cell responses? In this report we have analyzed degeneracy at the T cell level in the context of recognition of universal Th epitopes from diverse sources such as a viral, a bacterial, and a protozoan parasite protein (14–18) with no apparent sequence homology. At the bulk T cell level, three unrelated promiscuous T cell epitopic sequences were able to induce T cell responses in the context of multiple MHC class II molecules. Furthermore, a panel of T cell hybrids expressing a limited repertoire of TCR Vβ was also able to manifest a high level of flexibility in TCR recognition. However, the recognition of “promiscuous T cells” was highly specific, as none of the hybridomas was able to recognize the MHC class II-restricted (I-AB/Eβ) peptide SWM106–118. Our findings clearly demonstrate a high level of TCR plasticity, which somehow appears to be restricted to the recognition of universal MHC binders.

Our findings imply that unsuspected cross-reactivities may play an important role in the generation of T cell memory, the pathogenesis of autoimmune diseases, and possibly in a wide range of postimmune responses to infectious pathogens. The ability of structurally dissimilar promiscuous peptides to mimic each other when bound to class II MHC molecules may also be important in understanding the development of the TCR repertoire during thymic selection.

Materials and Methods

Experimental animals

Six- to 8-wk-old female mice of various strains, viz., BALB/c (I-A5/Eβ), DBA/2 (I-A5/Eβ), C57BL/6 (I-A5/Eβ), and C3H (I-A5/Eβ) were purchased from the small animal facility, National Institute of Immunology.
Peptide synthesis, purification, and characterization

All peptides used in this study were synthesized with an automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) using Fmoc/2(1H-benzotriazole-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate chemistry. Purification of all synthetic peptides to a single peak was achieved by reverse phase HPLC on a μBondapack reverse phase C18 preparative column (Waters Associates, Milford, MA) using 0.1% trifluoroacetic acid/water with a 10–90% 40-min linear 0.1% TFA/acetonitrile gradient. The identity and purity of the peptides were confirmed by amino acid analysis (420A/130A derivatizer/HPLC after hydrolysis with 6 N HCl for 24 h in vacuo; Applied Biosystems) and electrospray mass spectrometry on a triple-quadrupole mass spectrometer equipped with an electrospray ion source (TSQ 700, Finnigan MAT, San Jose, CA). In some cases to confirm peptide identity, amino-terminal sequence analysis was accomplished by automated Edman microsequencing using a pulse liquid protein sequencer (477A peptide sequencer, Applied Biosystems). Lyophilized peptides were dissolved in ultra pure water and stored frozen at −20°C, and their serial dilutions in the culture medium were prepared immediately before each assay. The sequences of these peptide constructs are given in Table I.

Cell culture medium

For lymphocyte culture and assays, RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with heat-inactivated FCS, 2 mM l-glutamine, 25 mM HEPES, 1% antibiotic/antimycotic solution (100×), 0.1 mM MEM non-essential amino acid solution, 1% anti-pleuropneumonia-like organism agent as 100× Tylosin (Life Technologies, Gaithersburg, MD), 0.2% sodium bicarbonate, and 5 × 10−5 M 2-ME (Sigma). Media containing no FCS, 10% FCS, and 20% FCS are referred to as RP0, RP10, and RP20, respectively.

Cell lines and mAbs

For generation of the T cell hybridoma BW 5147 (α/β) the thymoma cell line as a fusion partner was obtained as a gift from Dominique Ruff Juy (Pasteur Institute, Paris, France). For the Ag presentation assay, the mouse B cell lymphoma cell line A-20 was obtained from Dr. Satyajit Rath (National Institute of Immunology). mAbs 14.4.4S (anti-I-Ed), 212.A1 (anti-I-Aδ), 14-FITC, 6-FITC), TR310 (anti-Vβ1), 8-FITC), MR10 –2 (anti-Vβ4-FITC), 20.6 (anti-Vβ2-FITC), KT4 (anti-Vβ4-FITC), RR4-7 (anti-Vβ6-FITC), TR310 (anti-Vβ7-FITC), F23.1 (anti-Vβ8-FITC), MR10–2 (anti-Vβ9-FITC), B21.5 (anti-Vβ10-FITC), 14–2 (anti-Vβ14-FITC), Goat anti-hamster IgG-PE and goat anti-rabbit IgG-FITC were purchased from Cedarlane Laboratories (Hornby, Canada).

Lymph node T cell proliferation assay

Mice were immunized with 70–100 μg of peptide/mouse in PBS in a 1/1 (v/v) emulsion with CFA (Difo, Detroit, MI) by injecting it s.c. at the base of the tail and in the hind footpad. After 10–12 days the draining lymph nodes (inguinal and popliteal) were removed, cell suspensions were washed three times with RPMI 0.15M and then cultured in a flat-bottom 96-well plate (Falcon; Becton Dickinson, Lincoln Park, NJ) at a concentration of 5 × 10^5 cells/well in RP10 medium. Peptide Ags were added at various concentrations (2.5–60 μg/ml, final concentration). All cultures were incubated at 37°C in a 5% CO2 in humidified atmosphere. As a positive control, Con A (Sigma) was used at a final concentration of 5 μg/ml, while HP-22 peptide was used at a final concentration of 20 μg/ml as a negative control. The cells were incubated with 1 μCi/well of [methyl-3H]thymidine (NEN-DuPont, Boston, MA) for the last 16 h of a 5-day culture. Then, the cells were harvested using a Micro Cell Harvester (Skantron Instruments, Sterling, VA), and the incorporation of radioactivity was assayed by liquid scintillation counting using a 1205 Betaplate counter (LKB Instruments, Gaithersburg, MD). The results were expressed as the mean counts per minute of triplicate or quadruplicate cultures. The final results were calculated as the stimulation index of the ratio of counts per minute in the presence of Ag to counts per minute in controls (without Ag).

Positive responses were accepted when the stimulation index was >2.0.

Generation and long term maintenance of the CT.3T3_(778–395)-specific CD4+ T cell line

BALB/c mice were immunized with 100 μg of CT.3T3_(778–395) peptide emulsified in CFA (1/1, v/v) and injected s.c. at the base of the tail and in the hind footpad. After 2 wk the draining lymph nodes were removed, single-cell suspension were prepared, and cells were washed three times with balanced salt solution. From a single-cell suspension of lymph node cells, CD4+ T cells were isolated by negative selection using an immunoadfinity column containing mAb to CD8+ and B cells (Isolec mouse CD4+ isolation kit, Pierce, Rockford, IL). The purity of recovered CD4+ T cells was always >90%, as determined by staining with FITC-labeled anti-mouse CD4 mAb (GK1.5) using flow cytometry.

The CD4+ T cell line was initiated in a 24-well plate (Falcon) by stimulating 5 × 10^5 CD4+ T cells with 2 × 10^6 gamma-irradiated (60Co source, 3000 rad) synigen splenocytes as APC in 2 ml of RP10 medium in the presence of CT.3T3_(778–395) peptide (30 μg/ml, final concentration) for 7 days. Viable cells were separated from dead cells by density gradient centrifugation on Lympholyte-M (Cedarlane) and then restimulated with gamma-irradiated syngen splenocytes in complete medium containing 50 U/ml of human IL-2 (Genzyme, Cambridge, MA) and CT.3T3_(778–395) peptide. Thereafter, cells were restimulated with gamma-irradiated syngen splenocytes, human rIL-2, and CT.3T3_(778–395) peptide. In this way cells were subjected to several cycles of stimulation at intervals of 10–14 days until the requisite number of cells was obtained for testing. The Ag specificity of this T cell line was tested using proliferation assay and quantification of IL-2 in culture supernatants as described below.

Proliferation assay using the CT.3T3_(778–395)-specific CD4+ T cell line

CD4+ T cells were used 6–7 days after passage in IL-2-containing medium. Before assay, cells were depleted of dead APC by density gradient centrifugation on Lympholyte-M (Cedarlane). All assays were conducted in 96-well flat-bottom culture plates (Falcon) incubated at 37°C in 5% CO2 in a humidified atmosphere. A total of 5 × 10^5 cells/well were cocultured with 3–5 × 10^6 cells/well of irradiated (3000 rad) splenic APC from syngen mice in RP10 medium in the presence of various concentration of peptide Ags. There were three sets of controls in the assay: 1) APC alone in medium without Ag, 2) APC plus T cells in medium without Ag, and 3)
T cells alone in medium with Ag but no APC. Both IL-2 production and proliferation were used to assess T cell stimulation in these cultures. For the quantification of IL-2 in the culture, 100 μl of culture supernatant were collected after 24 h of stimulation from each set and preserved at −20°C until the assay was performed. The IL-2 concentration was measured using a mouse IL-2 ELISA kit (Biotrak, Amersham, U.K.). The proportion of T cells was measured by addition of 1 μCi/well of [methyl-³H]thymidine (NEN-DuPont) for the last 16 h of a 90-h culture period. Incorporation of radioactivity was assayed by liquid scintillation counting as described above. The results were expressed as the mean counts per minute of quadruplicate cultures.

MHC blocking assay
For MHC blocking studies mAbs 14.4.4S (anti-I-E^d^), 212.A1 (anti-I-A^d^), and HB-75 (anti-H-2k) as hybridoma culture supernatant (10%, v/v) were included in proliferation assays at the time of initiation of peptide restimulation. As a control, anti-glycophorin mAbs was used. Peptide-specific proliferation of the CD4⁺ T cell line was measured as [³H]thymidine (NEN-DuPont) incorporation and quantification of IL-2 production in culture supernatant (collected after 24 h of culture) using the mouse IL-2 ELISA kit (Biotrak) as described above.

Generation of TT₃₈₀⁻₈₄₄-specific T cell hybridomas
T cell hybridomas against peptide TT₃₈₀⁻₈₄₄ were produced essentially as described previously in standard protocols (26) with few modifications. Briefly, a group of BALB/c mice was immunized with 70–100 μg of TT₃₈₀⁻₈₄₄ peptide/mouse in distilled water in a 1/1 (v/v) emulsion with CFA (Difco) by injecting the peptide s.c. at the base of the tail and in hind foot pad. After 14 days of priming, draining lymph nodes (inguinal and popliteal) were removed in ice-cold RPMI medium. After teasing, a single-cell suspension was obtained by passing the cells through a 70-μm pore size cell strainer (Falcon), washed three times in RPMI medium, and finally suspended in RPMI medium. A total of 5 × 10⁶ cells/ml of lymph node cells (LNCs) were stimulated with TT₃₈₀⁻₈₄₄ peptide (30 μg/ml) and human rIL-2 (30 U/ml) in RPMI medium at 37°C in 5% CO₂ in a humidified atmosphere. After 3 days, T cell blasts were purified by density gradient centrifugation on Lympholyte-M (Cedarlane) and cultured overnight with 10 U/ml of rIL-2. The following day, T cell blasts were fused to the thymoma BW 5147 (α/β²; fusion partner), a parental tumor cell line containing no genes coding for functional TCR α⁻β-chains (27), at a 1:1 ratio using polyethylene glycol-1500 (Roche, Indianapolis, IN). After 24 h hypoxanthine-aminopterin-thymidine (HAT) (Sigma) selection medium was added to select for BW T cell hybridomas. Cells were expanded into 24-well plates under HAT selection and then with RPMI medium containing hypoxanthine-thymidine (Sigma). Once produced, uncloned T cell hybrids from individual wells were expanded in RPMI medium containing hybridoma growth factors and analyzed for the expression of CD3 and CD4 by flow cytometry. The uncloned hybridomas expressing a high level of CD3/CD4 were further analyzed for IL-2 production in response to TT₃₈₀⁻₈₄₄ peptide presented by irradiated splenocytes as APCs from BALB/c mice. The hybridomas positive for IL-2 secretion were further cloned by limiting dilution (0.3–1 cell/well), and nine well-growing clones were selected for their Ag recognition and TCR Vβ gene expression.

Ag presentation assay with T cell hybridomas
Assays were performed essentially as previously described by Vignali and Strominger with slight modifications (28). In all assays B lymphoma cells (A-20) were used as APCs and labeled with a given peptide overnight before each assay. Briefly, T cell hybridomas (5 × 10⁵ cells/well) were stimulated with peptide-labeled A-20 cells (5 × 10⁵ cells/well) in RPMI medium in 96-well flat-bottom plates. Various concentrations of peptides, ranging from 1.25 to 160 μg/ml, were used. All cultures were performed in duplicate, and 100 μl of culture supernatants were collected 24 h later. IL-2 estimation was performed by ELISA using DuoSet mouse IL-2 ELISA development kits (R&D Systems, Minneapolis, MN) following the protocol provided by the manufacturer.

TCR Vβ gene usage by T cell hybridomas
TCR Vβ gene usage were determined by flow cytometry using a panel of fluorochrome-labeled mAbs. Naive BALB/c splenocytes and BW 5147 (α/β) cells were used as control cells, while, as an isotype control, anti-hamster IgG-PE and anti-rabbit IgG FITC were used during the assay.

For direct surface staining, 1 million viable cells were incubated at 4°C for 1 h with 1 μg of either PE- or FITC-conjugated anti-mouse mAbs in a total volume of 100 μl of staining buffer (PBS containing 1% FCS and 0.1% sodium azide). Cells were then washed (three times, 2000 rpm, 5 min), resuspended in 500 μl of staining buffer containing 1% paraformaldehyde (Sigma). CD3 expression was detected by indirect staining with hamster anti-mouse CD3 mAb (500A2) followed by PE-conjugated goat anti-hamster IgG mAbs. All flow cytometric analyses were performed on a FACS Calibur instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA). Samples were gated for live cells based on forward and side scatter parameter (10,000 events/sample) and were analyzed by using CellQuest software (Becton Dickinson).

Results
Cross-reactivity of promiscuous epitopes at the bulk T cell level To assess cross-reactivity among the three promiscuous T cell epitopes, four strains of mice, viz., BALB/c (I-A^d^I-E^d^), DBA/2 (I-A^d^I-E^d^), C57BL/6 (I-A¹I-E¹), and C3H (I-A¹I-E¹), were immunized and used in the lymphoproliferation assay as described in Materials and Methods. In the first instance these strains of mice were immunized with promiscuous Plasmodium falciparum peptide CS.T₃₇₈⁻₃₉₅ and the draining LNC were examined for their ability to proliferate to different peptides used in this study. Primed LNC efficiently responded to CS.T₃₇₈⁻₃₉₅, Iₐ₄₅⁻₆₀, TT₃₈₀⁻₈₄₄ peptide, and tetanus toxoid (Table II). No lymphoproliferation was observed when these LNC were stimulated with CS.T₃₅₉ or an unrelated 22-residue peptide (HP-22) derived from a major malaria P. falciparum protein called histidine-rich protein II. These results indicate that the cross-reactivity seen with the promiscuous peptides in this assay was specific to the sequences of these peptides.

Groups of mice of different strains were also immunized with either TT₃₈₀⁻₈₄₄ or Iₐ₄₅⁻₆₀, and the draining LNC were examined for their ability to proliferate in response to stimulation with the three promiscuous T cell epitopes. Regardless of the immunizing peptide, the primed LNC proliferated in response to CS.T₃₇₈⁻₃₉₅, TT₃₈₀⁻₈₄₄, Iₐ₄₅⁻₆₀, and tetanus toxoid, but, as observed above, not in response to the presence of the two control peptides, CS.T₃₅₉ and HP-22 (Table II). These control peptides, synthesized by the same procedures as other experimental peptides, were included as negative controls and to ensure that contaminants, if any, were not responsible for the observed cross-reactivity. Peptide CS.T₃₅₉ was based on the sequence of CS.T₃₇₈⁻₃₉₅ itself, whereas HP22 was based on another major malaria protein (histidine-rich protein II). Thus, immunization with any of the three promiscuous peptides produced cross-reactive T cell responses not only in response to stimulation with the individual peptides but also in response to tetanus toxoid. To determine whether immunization with tetanus toxoid also would induce a peptide-specific cross-reactive response, groups of two strains of mice, BALB/c and C57BL/6, were immunized with tetanus toxoid. In both strains of mice, the primed LNC proliferated significantly in response to stimulation with the individual peptides but not in response to the presence of the two control peptides, CS.T₃₅₉ or HP22. In all the above experiments control mice were immunized with PBS emulsified in CFA, but their LNCs gave no response to either the peptides or the protein stimulation, demonstrating and ruling out mitogenic contamination of the peptides (Table II).

CS.T₃₇₈⁻₃₉₅-specific CD4⁺ T cell line shows cross-reactivity with other promiscuous peptides
Having observed that the promiscuous Th peptides induced cross-reactivity at the bulk T cell level, we wanted to demonstrate this epitopic mimicry at the level of the peptide-specific CD4⁺ T cell line. To do this, BALB/c mice were immunized with CS.T₃₇₈⁻₃₉₅ peptide, and a peptide-specific CD4⁺ T cell line was generated and
maintained (see Materials and Methods). Upon in vitro stimulation, the CD4^+ T cell line responded efficiently individually to the presence of the three promiscuous peptides in a 90-h T cell proliferation assay (Fig. 1A). We found that the cell line did not respond to stimulation with the two control peptides, CS.T3scr and HP22. Consistent with the T cell proliferation data, we found that the level of IL-2 in the culture supernatants, collected after 24 h of stimulation with the individual promiscuous peptides, was 4- to 8-fold higher than the level of IL-2 produced by cells stimulated with CS.T3scr peptide (Fig. 1B).

Having observed cross-reactivity among the promiscuous peptides, we then asked whether well-known class II-restricted Th peptides would also cross-react with the promiscuous peptide-specific CD4^+ T cell line. To do this we synthesized two peptides with different class II restrictions, IA^bIE^d-restricted peptide SWM106-118 (29) and I-A^d-restricted peptide HEL46-61 (30) (Table I) and tested for their T cell specificity. In a classical lymph node T cell proliferation assay, as shown in Fig. 2, peptides SWM106-118 and HEL46-61 were both able to induce significant in vitro proliferation of LNCs from homologous peptide-immunized BALB/c and C3H mice, respectively (maximum counts per minute, 11,250 for SWM106-118 and 10,600 for HEL46-61 at 30 μg/ml peptide concentration), while only a background level of proliferation was observed upon stimulation with unrelated peptides HP22 and CS.T3scr. When the CS.T3_{378-395}-specific CD4^+ T cell line was stimulated with either of the two peptides (SWM106-118 and HEL46-61), as shown in Fig. 3A, no proliferation of T cells was observed, whereas in the same assay the homologous peptide (CS.T3_{378-395}) induced a significant amount of proliferation of T cells. We also assessed the production of IL-2 in the culture supernatants from the above experiments. Significant peptide-specific induction of IL-2 was observed upon stimulation with CS.T3_{378-395} peptide, while only a background level of IL-2 was produced in culture stimulated with HEL46-61 and SWM106-118 peptide (Fig. 3B). These results show that at the level of the CD4^+ T cell line, CS.T3_{378-395}-primed T cells specifically cross-react with heterologous promiscuous peptide sequences.

**Inhibition of peptide-specific proliferation with anti-MHC class II-specific mAbs**

To test whether the promiscuous peptide-induced proliferation was governed primarily by MHC class II pathways, we conducted the proliferation assays in the presence of anti-MHC class I and anti-MHC class II mAbs. As shown in Fig. 4, CS.T3_{378-395}-specific proliferation of the CD4^+ T cell line using irradiated syngenic APC was inhibited by anti-MHC II mAbs, whereas the presence of anti-MHC I mAb or the control anti-glycophorin mAb did not cause any inhibition. In fact, at a lower concentration of the peptide a higher degree of proliferation was observed upon addition of anti-H-2K^d and anti-glycophorin mAb compared with that when no mAb was added. IL-2 production in the culture supernatants collected from the above inhibition experiments was also measured, and the results (data not shown) further suggested that the MHC class II pathways were mostly responsible for the promiscuous peptide-specific proliferation of the T cells.

**Recognition of promiscuous Th peptides at the clonal T cell level**

To further analyze the cross-reactivity observed at the clonal T cell level, we generated T cell hybridomas by fusing TT_{830-844}-primed lymph node T cells to thymoma BW 5147 (α/β0) as described in Materials and Methods. After selection of the hybrids on HAT/ hypoxanthine-thymidine medium, we analyzed the uncloned hybridomas for the expression of CD3 and CD4 molecules. Uncloned hybrids expressing a high level of CD3^+ /CD4^+ were able to respond to the peptide TT_{830-844} (at 30 μg/ml of peptide) presented by irradiated BALB/c splenocytes as APCs. Further, we performed the limiting dilution (0.3 and 1 cell/well), and nine well-growing hybrids for the expression of CD3 and CD4 molecules. Recognized promiscuous Th peptides at the clonal T cell level

### Table II. Cross-reactivity among promiscuous T cell epitopes at the bulk T cell level

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>In Vitro-Stimulating Ags SI^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse Strains</td>
</tr>
<tr>
<td>CS.T3_{378-395}</td>
<td>BALB/c (2522 ± 692)</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 (3628 ± 562)</td>
</tr>
<tr>
<td></td>
<td>DBA/2 (1246 ± 178)</td>
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<td></td>
<td>C3H (4640 ± 832)</td>
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<tr>
<td>1A_{45-60}</td>
<td>BALB/c (1672 ± 356)</td>
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<td></td>
<td>C3H (2725 ± 724)</td>
</tr>
<tr>
<td>TT_{830-844}</td>
<td>BALB/c (1326 ± 226)</td>
</tr>
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<td>C3H (2725 ± 724)</td>
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<td>Tetanus toxoid</td>
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<td>DBA/2 (5016 ± 899)</td>
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<tr>
<td></td>
<td>C3H (1600 ± 250)</td>
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</tbody>
</table>

^a In vitro lymphoproliferation of LNC from peptide-primed mouse strains on stimulation with CS.T3_{378-395}, TT_{830-844}, 1A_{45-60}, CST3_{sc}, and tetanus toxoid. Ags. This experiment was repeated three times with reproducible results. Data are from one representative experiment. Maximum value of SI is presented here. Immunogens are defined in Table I.

^b SI, Stimulation index (mean cpm in presence of Ag/mean cpm in controls (medium only)).
presentation assay, as shown in Fig. 5A, five clones, E.1.2, C.1.8, C.1.11, D.1.11, and D.2.6, were able to recognize the homologous peptide TT830–844 (maximum IL-2 secretion, 1700, 5500, 1400, 1800, and 2800 pg/ml, respectively). However, four clones, C.1.8, C.1.11, D.1.11, and D.2.6, also recognized heterologous promiscuous Th epitopes. Two hybridoma clones were able to recognize only two heterologous epitopes; clone C.1.8 responded to CS.T3378–395 and PfCSP 331–350 maximum (4300 and 3000 pg/ml IL-2) at concentrations of 80 and 160 μg/ml, respectively, while clone C.1.11 was able to secrete maximum IL-2 at concentrations of 20 and 160 μg/ml with CS.T3378–395 (1000 pg/ml) and IA45–60 (1100 pg/ml), respectively. The clone D.1.11 responded to the peptide CS.T3378–395 (maximum IL-2, 1600 μg/ml) and IA45–60 (maximum IL-2, 2500 pg/ml) at concentrations of 80 and 160 μg/ml, respectively, while peptides myelin basic protein (MBP)152–165 and PICSP331–350 induced maximum IL-2 production (2500 and 1500 pg/ml) at concentrations of 80 and 160 μg/ml, respectively. Interestingly, clone D.2.6 was able to recognize all six heterologous promiscuous peptides significantly and therefore represents a highly degenerate T cell hybridoma clone in this study.

However, the affinity of peptide recognition by this clone was different for the different peptides; maximum IL-2 secretion induced by CS.T3378–395 and IA45–60 was at 40 μg/ml of peptide, HA306–318 and MBP152–165 induced maximum response at 80 μg/ml, while PICSP345–362 and PICSP331–350 induced maximum IL-2 secretion at a higher concentration (160 μg/ml).

Surprisingly, the remaining four clones (D.1.10, F.1.6, G.1.9, and E.1.4) were not able to recognize the parent peptide TT830–844, but could recognize other heterologous promiscuous peptides significantly (Fig. 5B). This seems to be a heteroclitic response, where modified peptides bind to TCR more efficiently than the parent peptide. The clone D.1.10 responded to only IA45–60 and MBP152–165 at the concentration of 80 μg/ml, while clone F.1.6 was able to recognize MBP152–165 and PICSP331–350 with a high level of IL-2 secretion (maximum IL-2, 2600 and 2200 pg/ml at 40 and 10 μg/ml, respectively) and HA306–318 with a moderate level of IL-2 secretion (250 pg/ml IL-2 at 40 μg/ml). Maximum responses of clone G.1.9 to peptides CS.T3378–395, and IA45–60 were at 40 μg/ml of peptide, while maximum responses to HA306–318, and MBP152–165 were observed at 80 μg/ml. Unlike clone D.2.6, none of the four clones (D.1.10, F.1.6, G.1.9, and E.1.4) were able to recognize all six heterologous peptides. Only clone E.1.4 was able to respond to five unrelated sequences at various concentrations of peptides (CST378–395, MBP152–165, and
PfCSP 331–350 at 80–160 μg/ml, while IA 45–60 and HA 306–318 at 20–40 μg/ml). Recognition of the various unrelated promiscuous epitopes used in this study is specific to their primary structures to the extent that IA d-restricted Th epitope SWM 106–118 was not recognized at any concentration of peptide (1.25–160 μg/ml) by any of the nine T cell hybridoma clones (Fig. 5).

TCR Vβ gene usage of T cell hybridomas

From the above findings, it is clear that there is a high level of degeneracy at the T cell level in the recognition of multiple MHC class II allele binder peptides. We next investigated TCR Vβ usage of these T cell hybridoma clones. Analysis of TCR Vβ gene expression was performed by using flow cytometry after staining with fluorochrome-labeled commercially available anti-mouse TCR Vβ mAbs. The coreceptor CD3/CD4 staining revealed that all nine clones were strongly positive for CD3/CD4. Limited usage of TCR Vβ repertoire was indicated by the expression of Vβ4, Vβ8, and Vβ14 TCR (Table III). The results show that clones bearing the same TCR differed in their peptide recognition. E.1.2, C.1.8, and C.1.11 all expressed Vβ8, but did not recognize similar peptides. However, Vβ14+ T cell hybridoma clones were able to accommodate the maximum number of peptides (for example, clone D.2.6 recognized all; Table III). This is quite possible, as there may be differences in their Vα- or Jβ-chain usage, and a T cell can respond to unrelated peptides differently by selectively activating some pathways but not others (32).

Discussion

The use of T cell determinants that can be recognized in the context of several class II MHC molecules in place of carrier proteins to enhance the immunogenicity of B cell epitope sequence is currently being investigated in the design of subunit vaccines (24, 33). In an earlier study we observed that T cells from mice immunized with a well-known carrier protein, tetanus toxoid, could be stimulated not only with peptides containing the helper sequences from tetanus toxoid itself, but also with an unrelated, promiscuous Th epitope from the circumsporozoite protein of *P. falciparum* (S. K. Joshi and V. S. Chauham, unpublished observations). This prompted us to ask whether promiscuous Th epitopes would generally produce cross-reactive T cell responses. Initially, three well-characterized epitopes of three different origins were selected for this study (14–16). Our results showed that immunization with any of the three promiscuous peptides (CS.T378–395, IA 45–60, and TT 830–844) produced cross-reactive cellular responses in lymphoproliferation assays, whereas a peptide corresponding to the scrambled sequence of one of the promiscuous peptides (CS.T378–395) did not show any reactivity in these assays, like another unrelated Th malaria peptide sequence. Although the three promiscuous peptides are well-characterized Th epitopes, we wanted to confirm that the cross-reactivity seen at the bulk T cell level was due to class II
MHC pathways, so we developed a peptide (CS.T3 378–395)-specific CD4+ T cell line from BALB/C mice immunized with CS.T3 378–395. The results of lymphoproliferation assays and IL-2 analysis with the CD4+ T cell line suggested that cross-reactive responses were mostly due to the Th pathways. The results of MHC blocking assay also provided evidence that the CS.T3 378–395 peptide was recognized in association with MHC class II molecules. These data suggest that not only are these promiscuous Th epitopes capable of binding several different class II MHC molecules, but they also possess structural features in their sequences that allow the peptide-MHC complex to be recognized by the TCR to produce cross-reactive T cell responses. Although two appropriate control peptides, prepared by the same procedures as the experimental peptides, did not show any cross-reactivity with promiscuous peptide-specific T cells, we wondered whether the observed cross-reactivity was a chance observation. Although the three cross-reactive promiscuous peptides were chosen from different sources, some structure similarities, such as the presence of positively charged residues in the middle of their sequences, could be detected. To address this question, and to discount such a possibility, four more promiscuous peptides from different sources were included at this stage in this study. Our results with these peptides clearly indicated that at the bulk T cell level promiscuous peptides indeed produced cross-reactive T cell activation.

To further establish the cross-reactivity among the promiscuous epitopes at the clonal T cell level, we generated T cell hybridoma clones against one of the three initially selected peptides (TT 830–844). Results with these clones further confirmed our earlier observation at the bulk T cell level and with the peptide-specific CD4+ T cell line. What could be the basis for such extensive cross-reactivity at the T cell level among these peptides, although promiscuous in their recognition of the class II molecules, from different sources and with different primary structures? In earlier studies it was found that a certain degree of homology between T cell epitopes was essentially required for the cross-reactive T cell activation, as in the case of hemagglutinin matrix protein and hemagglutinin (34). Surprisingly, however, an increase in homology abolished the cross-reactivity, suggesting that increasing homology alone may not be related to cross-reactivity. Similarly, while eight identical residues were required in the recognition of a human HLA-DQ-restricted clone by two different T cell epitopes from mycobacterial heat shock protein 65 and the human heat shock protein 60 (35), only two unique residues were sufficient for the recognition of an HLA-DR1-restricted T cell clone by a peptide from the influenza virus hemagglutinin (36). Extreme cases of T cell cross-reactivity involving peptides without any apparent sequence homology have also been recently reported (2–12, 37). For example, Bharadwaj et al. observed degenerate recognition of a chimeric peptide based on MBP by cloned T cells (2). In another study several structurally dissimilar viral and bacterial peptides stimulated MBP-specific T cell clones (4). Likewise, Hagerty and Allen (12) identified two structurally dissimilar, but cross-reactive, T cell epitopes within a single human protein, human α1-antitrypsin. These reports and the results of the present study suggest that there is considerable cross-reactivity, such that one TCR is able to recognize a number of different peptides that do not necessarily share strong sequence homology.

The cross-reactivity of the T cell response to the peptides described here represents functional degeneracy at two levels, and in this regard is somewhat different from the examples mentioned above. The first level of degeneracy of peptide recognition is at the level of MHC class II binding itself, and although known for some time (14–24), the structural basis for the promiscuous recognition is not yet clear. Structural analyses of single peptides bound to MHC class II molecules have shown that in these peptides, ranging from 13 to 25 residues, the contact is essentially made by a central core of nine residues, as in the case of class I molecules, with the flanking residues on either sides spilling out over the ends of the binding site (38). There is extensive hydrogen bonding between the conserved elements of the groove and the backbone of the peptide, and even though binding sites are open at both ends, the backbone interactions result in tight structural constraints upon the class II binding peptides (38). Given this, a wide range of different peptides may be expected to bind with class II molecules, and the unfavorable interactions between amino acid side chains and MHC can be countered and stabilized by the wide network of backbone hydrogen bonding with MHC class II molecules (reviewed in Ref. 39). It is still not clear how a given promiscuous peptide binds to different class II MHC molecules. For example, Kilgus et al. (40) showed that distinct sites on the CS.T3 378–395 sequence interact in different ways with the three DR molecules analyzed. On the other hand, Panina-Bordignon et al. (15) showed that the promiscuous peptide TT 830–844 interacts in the similar way with different DR molecules, possibly by binding to the conserved DR residues. Chicz et al. (41) suggested that the ability of peptides to bind multiple MHC alleles must be dependent on the composition and location of several key amino acids within the primary structure, which led to the hypothesis that rigid allelic-specific motifs for the class II molecule do not exist, thus permitting a broad binding specificity.

Our results at the bulk T cell level, with the peptide-specific CD4+ T cell line, and with T cell hybridoma clones suggested a high level of degeneracy of recognition of the promiscuous peptide-MHC complexes by the TCR. While a certain degree of specificity for the MHC-bound peptide complexes is to be expected, there is now a large body of evidence indicating that individual TCRs can cross-react with many different peptide Ags bound to the same MHC molecule. Indeed, it has been proposed that a high level of cross-reactivity is an essential feature of the TCR (reviewed in Ref. 13). How are these structurally unrelated peptides recognized by the same T cells?

Structural analyses of the TCR-peptide-MHC complexes determined to date indicate that the peptide cross-reactivity can be produced in at least two ways: 1) the TCRs focus on only a few amino acid side chains of the peptide and can accommodate peptides with other side chains depending on the size and surface chemistry of the TCR contact surface; and 2) structural flexibility of the CDR3 loops that contact the peptide allow a degree of accommodation of binding to multiple peptide ligands (42–44). It appears that binding of the peptide/MHC complex to TCR in a diagonal orientation leads to TCR interaction with the relatively small area of peptide surface (one or two amino acids) that is not buried in the MHC molecule. Kinetic and thermodynamic studies also provide evidence that the TCR and/or peptide-MHC have flexible binding surfaces that stabilized upon binding and suggest that conformational flexibility may contribute to cross-reactivity in Ag recognition, as observed in the present study (45). On the other hand, in a study in which a single T cell clone could recognize at least five different peptides, it was suggested that the TCR may have multiple sets of contact residues for different peptide/MHC ligands, binding to any one of which can trigger the cell, and/or that the TCR could interact with the peptide/MHC complex in more than one orientation (9). It is therefore likely that several peptide sequences may satisfy the requirements of TCR recognition once they have crossed the hurdle of MHC binding with appropriate affinity. This may partly explain the cross-reactivity of the promiscuous peptides observed in the present study. However, at the same time we found that two different Th peptides of well-defined genetic restriction (HEL 46–61 and SWM 106–118), which bind to the
corresponding IA and IE class II molecules, respectively, did not show any cross-reactivity with the CS.T3 378–395-specific CD4+ T cell line. Similarly, none of the TT 830–844-specific hybridoma clones was able to recognize IAα-restricted SWM 106–118 Th epitope. These results suggest that the flexibility of the TCR recognition observed in the present study is somehow restricted to MHC-promiscuous peptide complexes, and it appears to be a specific recognition. Since we used only two genetically restricted Th peptides in this study the possibility...
of the promiscuous peptide-specific T cell recognition of other Th sequences cannot be ruled out.

For T cell activation, interactions of TCR with the peptide-MHC complex have a low affinity with $K_d$ values in the range of 1 to 90 $\mu$M (46). For many peptide-MHC complexes the repertoire of TCR that may achieve this affinity is expected to be quite large. To address this question in the present study we used TT830–844-specific T cell hybrids. Several T cell hybridoma clones were obtained, albeit they were limited in their usage of the V$\beta$. Nine hybridoma clones that we studied were found to bear only three V$\beta$ (V$\beta$4, V$\beta$8, and V$\beta$14). Our results show that four clones, D.2.6 and D.1.11 (V$\beta$14), and C.1.8 and C.1.11 (V$\beta$8) cross-reacted with different promiscuous peptides, whereas one T cell clone, D.2.6 (V$\beta$14$^+$), recognized all the peptides. Surprisingly, four other T cell hybridoma clones, E.1.4 and D.1.10 (V$\beta$4), and G.1.9 and F.1.6 (V$\beta$14), did not significantly react with the

Table III. TCR V$\beta$ gene usage and peptide recognition by T cell hybridomas

<table>
<thead>
<tr>
<th>Hybridoma Clones</th>
<th>Coreceptor Expression</th>
<th>TCR V$\beta$ Gene Usage</th>
<th>Peptide Recognition</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.1.2</td>
<td>CD3/CD4</td>
<td>V$\beta$8</td>
<td>TT830–844</td>
</tr>
<tr>
<td>C.1.8</td>
<td>CD3/CD4</td>
<td>V$\beta$8</td>
<td>TT830–844, CS.T378–395, PicSP331–350</td>
</tr>
<tr>
<td>D.1.11</td>
<td>CD3/CD4</td>
<td>V$\beta$14</td>
<td>TT830–844, CS.T378–395, 1A45–60, MBP152–165, PicSP331–350</td>
</tr>
<tr>
<td>C.1.11</td>
<td>CD3/CD4</td>
<td>V$\beta$8</td>
<td>TT830–844, CS.T378–395, 1A45–60</td>
</tr>
<tr>
<td>E.1.4</td>
<td>CD3/CD4</td>
<td>V$\beta$4</td>
<td>CS.T378–395, 1A45–60, HA306–318, MBP152–165, PicSP331–350</td>
</tr>
<tr>
<td>G.1.9</td>
<td>CD3/CD4</td>
<td>V$\beta$14</td>
<td>CS.T378–395, 1A45–60, HA306–318, MBP152–165</td>
</tr>
<tr>
<td>F.1.6</td>
<td>CD3/CD4</td>
<td>V$\beta$14</td>
<td>HA306–318, MBP152–165, PicSP331–350</td>
</tr>
<tr>
<td>D.1.10</td>
<td>CD3/CD4</td>
<td>V$\beta$4</td>
<td>1A45–60, MBP152–165</td>
</tr>
</tbody>
</table>

* Peptides are defined in Table I.
homologous peptide (TT<sub>830–844</sub>) itself as efficiently as they recognize other promiscuous peptides, suggesting a type of heteroclite response with respect to these peptides. It is not clear to us how this is achieved, but a possible explanation could be that in these peptides a TCR contact residue is replaced with an amino acid that is capable of even stronger interactions with the TCR (47). Such observations have also been recently reported by other workers (48, 49).

The ability of various structurally dissimilar peptides to fully activate the same T cell is not surprising. The general binding mode between the TCR-MHC peptide complex, which clearly supports cross-reactivity, is supposed to be part of the positive selection process in thymus, in which the T cells with low affinity for self MHC molecules are selected and the T cells with high affinity for their ligands are deleted (50, 51). Recent studies have shown that the T cells that have previously interacted with Ags can recognize a variety of ligands (52, 53). Consistent with this idea it was found that prior immunity to the virus can provide some level of protective natural immunity to an unrelated infectious agent (54). It is also realized that interactions not only with environmental Ags, but also with self-peptides may contribute to maintaining T cell memory in the absence of persistent Ags (4). Cross-reactive stimulation has been implicated in the maintenance of T cell memory in malaria, and cross-reactive stimulation among malaria Ags and some other common immunogens has been attributed as the main reason for the observation that T cells from nonexposed donors proliferate in response to malaria Ags (55, 56).

Finally, the results of this study show that several peptides that bind promiscuously to MHC class II molecules also show degeneracy of Ag recognition at the TCR level, and the observed cross-reactivity appears specific to their sequences. Our results, however, do not allow us to speculate whether and how the promiscuous T cell epitope cross-reactivity is involved in shaping the T cell repertoire in both thymus and periphery. It needs to be evaluated whether the cross-reactivity of the promiscuous Th epitopes can be exploited in connection with synthetic peptide-based vaccination strategies. Prior vaccination with a carrier protein to overcome the genetic restriction and to enhance the immunogenicity of peptide Ags has been reported (57). On the other hand, however, recent studies have indicated that molecular mimicry involving a given microbial epitope and a pool of cross-reactive self peptides could have important implications for the pathogenesis of autoimmune diseases, suggesting a link between immune responses to infectious agents and autoimmunity (58). Cross-reactive responses among promiscuous Th epitopes from different origins should be investigated for their role and use in modulating T cell responses in general.

Acknowledgments

We thank Prof. Herman N. Eisen for critical review of the manuscript, and Prof. Harald von Boehmer for valuable suggestions. We are grateful to Dr. S. Rath for helpful discussion, Dr. V. Bal for providing anti-MHC class I and class II mAbs, and Dr. D. Blackall for providing anti-glycoporin mAb. We also thank Drs. Ratanrami Joshi and Ashima Bharadwaj for their help with peptide synthesis and purification, Narendra Singh Negi for animal management, and A. C. Alexander for irrigation of splenocytes at Central Instrument Facility, Jawaharlal Nehru University (New Delhi, India).

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