IDENTICAL PEPTIDES RECOGNIZED BY MHC
CLASS I– AND II–RESTRICTED T CELLS

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Several lines of evidence suggest that the mechanism of T cell recognition of antigen is similar in both class I and class II MHC-restricted systems. First, the TCR molecules that recognize antigen in the context of either the class I or class II MHC molecules are assembled from the same set of germline variable region genes (V_α-J_α and V_β-D_β-J_β) (1). Furthermore, several groups have demonstrated that the same V region gene can be used by different T cell clones that have MHC restriction to different classes, either class I or II (2–5). Second, both the class I and II MHC molecules have functional and structural similarities. Guillet et al. (6) demonstrated that class II MHC molecules have a single antigen binding site based on functional analysis of antigen competition. The crystal structure of a class I (HLA-A2) molecule also suggests a single antigen binding site that is a cleft between two α helices located at the NH_2 terminus of the molecule (7). In addition, a model of the class II ($I-A^k$) structure proposes tertiary structural homology with the class I molecule, including a single antigen binding site (8). Thus, although the class I and II MHC molecules are different proteins and have primary sequence variability, the tertiary structure of both the class I and II molecules is predicted to be similar. Third, short synthetic peptides encompassing immunodominant regions of a protein can replace protein antigen in both class I and II–restricted T cell responses. Class I–restricted T cells lyse histocompatible target cells incubated with the appropriate peptide (9–14), and class II–restricted T cells proliferate and secrete lymphokines in response to histocompatible APCs plus peptide (15–18). Furthermore, two groups using different methods analyzed immunogenic peptides and identified structural motifs common to both class I and II–restricted peptides (14, 19). Thus, the trimolecular interactions between TCR, MHC molecule, and antigen have many similarities between the class I and class II systems.

Materials and Methods

Mice.   6–10-wk-old mice of the CBA, BALB/c, C57/B6, A/J, B10.A, B10.A(2R), B10.A(4R), B10.A(5R), and A.TL strains were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.GD mice were a gift of Dr. Don Schreffler (Washington University, St. Louis, MO). All mice were maintained in microisolator cages.

Peptides.   All peptides were synthesized by the solid phase method of Merrifield using an

automated peptide synthesizer (No. 430A; Applied Biosystems,) as previously described (20). Peptides were desalated on a G-25 column (2.5 × 90 cm) equilibrated with 4 M glacial acetic acid and then lyophilized twice after resuspension in distilled water. The purity of all peptides was determined by amino acid analysis.

Viruses.   Influenza virus strains A/NT/60/68 and A/Jap/57 were grown in the allantoic cavity of 10-d-old embryonated chicken eggs and stored as infectious allantoic fluid (21).

Lymph Node Proliferation.   Mice were immunized subcutaneously with 50 μg of the appropriate peptide in CFA and 7-10 d later popliteal, inguinal, and pararotic lymph nodes were removed. Lymph node cells from three mice of each strain were pooled and then split into six equal aliquots, which were incubated with the appropriate mAb for 1 h on ice, washed, and incubated with rabbit complement (Pel-Freeze Biologicals, Rogers, AR) at 1:10 plus mouse anti-rat κ (MAR18.5) (22). Cytotoxicity index based on trypan blue dye exclusion of a typical experiment was: no mAb, 5%; anti-CD4 (GK1.5, reference 29), 35%; anti-CD8, (AD4, reference 30), 24%; anti-Thy-1 (13.4, reference 31), 64%; anti-ARS (45-112, reference 32), 6%; complement control, 7%. All mAbs were prepared as ascites and used at 1:100 dilution. 6 × 10^5 lymph node cells plus 2 × 10^5 irradiated syngeneic splenic APCs cells were cultured in 100 μl in one-half area wells in microtiter plates with the indicated concentration of peptide for 48 h. 8 h before harvesting 1 μCi per well of [^{3}H]thymidine was added. Cells were harvested with an automated cell harvester (Skatron Inc.) and [^{3}H]TdR incorporation was measured by scintillation counting.

T Cell Hybridomas.   Hybridomas were prepared as previously described (23). Briefly, mice were immunized subcutaneously with 50 μg peptide in CFA and 7-10 d later cells from draining lymph nodes were purified and cultured at 4 × 10^6/ml with 10 μg/ml peptide for 2 d. Viable cells were purified by Ficoll-Metriozate gradient centrifugation and fused with an equal number of BW5147 cells using 0.5 ml 50% polyethylene glycol 1500 in RPMI. Hybridomas were assayed for antigen-specific reactivity by analysis of IL-2 secretion. 5 × 10^4 hybridomas plus 5 × 10^4 APCs plus 10 μg/ml of peptide were cultured for 24 h in microtiter plates. 50 μl of supernatant was added to 10^4 of the IL-2-dependent cell line CTLL.2 cells for 20 h, then 1 μCi [^{3}H]TdR per well was added per well for 4 h. Cells were harvested with an automated cell harvester and thymidine incorporation was measured by scintillation counting (23).

Cytotoxicity Assay.   Mice were immunized intranasally with 10 hemaglutinin units (HAU)[1] of influenza A virus and 2-4 wks later spleen cells were harvested and cultured at 10^6/ml with 2 × 10^5 virus-infected syngeneic spleen cells previously irradiated in a cesium irradiator (2,000 rad). After 5 d cells were harvested and purified in Ficoll-Metrizoate. The effector cells were added to target cells incubated in 0.5 mCi of ^{51}Cr plus 100 HAU influenza virus when indicated for 2 h and washed four times. 2 × 10^4 target cells were added per well in U-bottomed microtiter plates. Target cells were El-4 (H-2^b), P815 (H-2^d), and BW5147 (H-2^k). After a 4-h incubation period supernatants were harvested with a supernatant collection system (Skatron, Inc.) and counted with a gamma counter (Pakcard Instruments, Specific Cr release was calculated as: Specific Cr release = (experimental − spontaneous counts)/(total − spontaneous counts).

Results

Our experiments test the hypothesis that class I and II–restricted peptides are functionally interchangeable, based on the assumption that immunogenic peptides have similar structural motifs. Mice of three different H-2 haplotypes were immunized with a peptide from influenza nucleoprotein composed of residues 365-80 (NP365-80), which was identified previously in a class I–restricted cytotoxic response (9). Lymph node cells from immunized mice were assayed for T cell proliferation. A/J, B10.A, and B10.A(4R) mice demonstrated a positive proliferative response; however, C57/B6, BALB/c, and B10.A(5R) were negative (Table I). Townsend et al. had

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[1] *Abbreviation used in this paper:* HAU, hemagglutinin units.

TABLE I

NP365-80 Lymph Node Proliferation

Strain	H-2 KAED	Response NP365-80
C57/B6	bbbb	–
BALB/c	dddd	–
B10.A	kkkk	+
A/J	kkkd	+
B10.A(4R)	kkbb	+
B10.A(5R)	bbkd	–

4×10^5 lymph node cells were incubated with NP365-80 or control peptide at concentrations from 100 to 0.03 μM for 48 h. 1 μCi of [^{3}H]TdR per well was added for the last 8 h of culture, and cells were harvested with an automated cell harvester, and thymidine incorporation was measured by scintillation counting. Stimulation index (positive cpm/background cpm) was greater than seven for all positive responses.

previously shown that NP365-80 is class I D^b restricted (9). In our experiments the C57/B6 strain that expresses D^b did not demonstrate a proliferative response, whereas the A/J and B10.A strains that express the H-2 D^d and D^k alleles, respectively, were positive. These results suggest that the proliferating cells were not class I restricted. The three responding strains A/J, B10.A, and B10.A(4R) (but not C57/B6 or BALB/c) only share the class II I-A^k allele further suggesting that the proliferative T cell response to NP365-80 is class II restricted.

To test the generality of this result, we synthesized four additional murine class I-restricted peptides including two peptides from influenza nucleoprotein (NP50-63 [9] and NP147-158 [8]) and two peptides from influenza hemagglutinin (HA202-21 and HA523-45 [13]), immunized three strains of mice, and subsequently assayed lymph node proliferative responses. In this experiment specific T cell subsets were killed with complement plus mAbs that recognize the CD4, CD8, or Thy-1 to determine which T cell subset was proliferating. In Fig. 1, *a-e*, CD4$^+$ but not CD8$^+$ T cells proliferated in response to all five different peptides NP50-63, NP147-58, NP365-80, HA202-21, HA523-45 defined previously in class I-restricted cytotoxicity assays. Mice of all three haplotypes (H-2^{b,d,k}) responded to HA202-21; however, for the other four peptides only one of the three haplotypes demonstrated a positive response (data from nonresponding haplotypes not shown). The failure to respond to a specific peptide by the majority of H-2 haplotypes demonstrates the specificity of the T cell responses. Because CD4$^+$ T cells are class II restricted, these results provided further evidence that the previously defined class I-restricted peptides also generated a class II-restricted T cell response. Concordant results were obtained when lymph node cells were analyzed for IL-2 secretion (not shown).

To further demonstrate the class II MHC restriction of the proliferative responses, T cell hybridomas were made from mice immunized with NP365-80 and screened for IL-2 secretion after culture with peptide plus fibroblast L cells transfected with either I-A^k or I-E^k as APCs (gifts of R. Germain, National Institutes of Health, Bethesda, MD). Of 169 hybrids specific for NP365-80 derived from two separate fusions in A/J and B10.A mice, 162 were I-A^k restricted. Seven I-E^k-restricted hybrids were also identified (Table II). Thus, CD4$^+$ class II-restricted T cells can
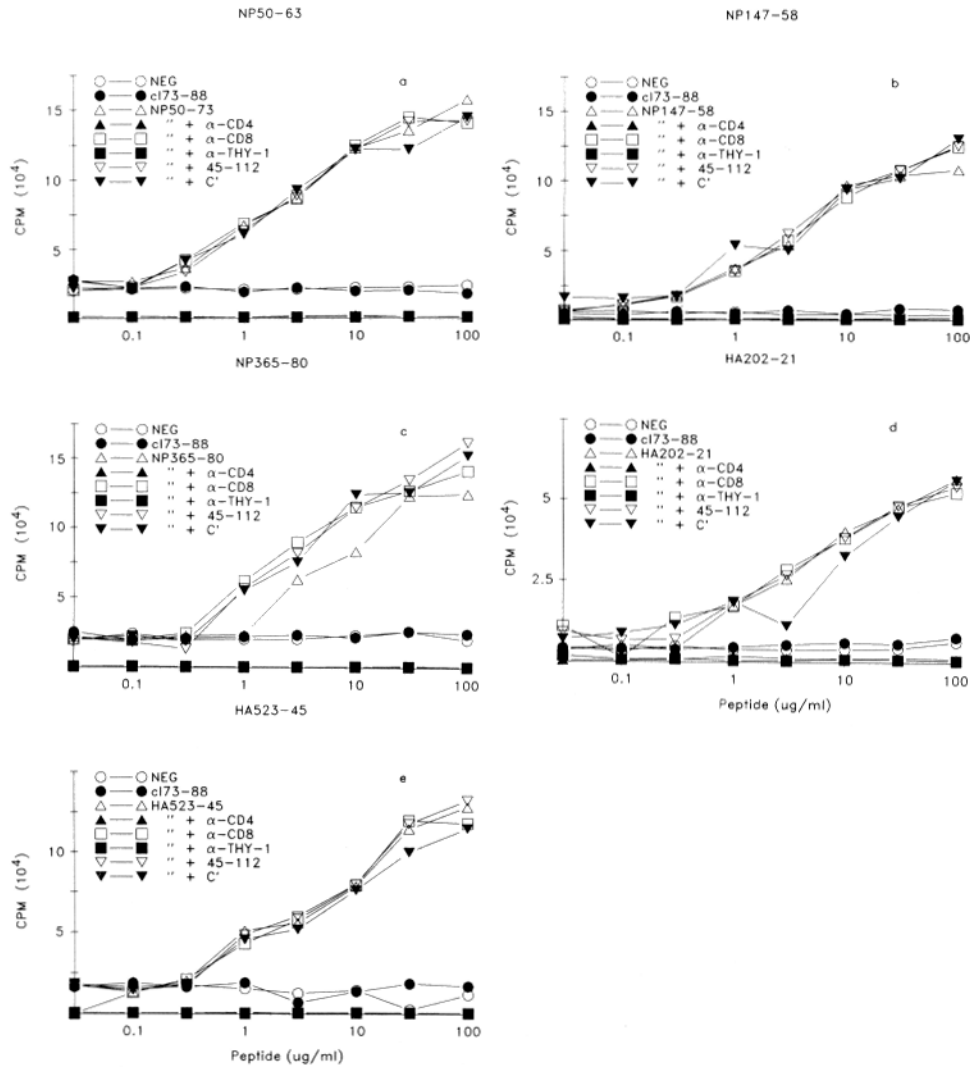
FIGURE 1.   Lymph node proliferation analysis of peptides previously identified in murine class I–restricted cytotoxicity assays. Peptides (a) NP50-63 (11), (b) NP147-58 (12), (c) NP365-80 (9), (d) HA202-21 (13) (e) HA523-45 (13) were synthesized by the solid phase method of Merrifield using an automated peptide synthesizer (No. 430A; Applied Biosystems) (20). Six aliquots of lymph node cells from each strain were assayed: no peptide and no mAb or complement (C′) (O), cI73-88 (peptide 73-88 from λ repressor cI) with no mAb or C′ (●), peptide and no mAb or C′ (△), peptide plus anti-CD4 mAb (GK1.5) (29) plus C′ (▲), peptide plus anti-CD8 mAb (AD4, reference 30) plus C′ (□), peptide plus anti-Thy-1 mAb (13.4, reference 31) plus C′ (■), peptide plus antiarsonate (ARS) mAb (45-112, reference 32) plus C′ (▽), peptide plus C′ without mAb (▼). Mouse strains were CBA (a, c, and e), BALB/c (b), and C57/B6 (d).

respond to NP365-80, a peptide previously identified as class I D^b–restricted peptide, in the context of the class II I-A^k molecule, confirming that the same peptide was recognized in both a class I and class II–restricted manner.

Next we tested the same five peptides in cytotoxicity assays to confirm their class

Table II

NP365-80-specific T Cell Hybridomas

	APCs	
Strain	A^k	E^k
A/J	131	7
B10.A	38	0
Totals	169	7

Mice were immunized subcutaneously with 50μg of NP365-80 in CFA and 7-10 d later draining lymph nodes were removed and cells were stimulated with 10 μg/ml NP365-80 at 4 × 10⁶ cells/ml for 48 h. Cells were then purified with Ficoll-Metrizoate centrifugation and fused with BW5147 using PEG1500 as previously described (23). 5 × 10⁴ hybridoma cells plus 5 × 10⁴ APCs were incubated with 10 μg/ml NP365-80 for 24 h. 50 μl supernatant was harvested and added to 10⁴ CTLL.2 IL2-dependent cells for 24 h. 1 μCi [³H]TdR per well was added during the last 4 h of culture. Cells were harvested with an automated cell harvester and thymidine incorporation was analyzed by liquid scintillation counting.

I restriction. Mice were immunized with influenza A, and after secondary stimulation in vitro, spleen cells were assayed for cytotoxicity with histocompatible target cells incubated with appropriate peptide or infected with influenza virus. Because all the tumor cell lines used as target cells express class I but not class II MHC molecules, cytotoxicity must be class I restricted. As expected, infected target cells were specifically lysed in each case. Furthermore, all five peptides also induced cytotoxicity, although the percent specific ^{51}Cr release was greater for virally infected target cells than for target cells in the presence of peptide (Fig. 2, *a-e*). This could be due to the presence of additional class I epitopes on the virally expressed proteins or to increased density of antigen on the infected target cells. Nevertheless, all five peptides can clearly be recognized by T cells in a class I-restricted assay.

Structural homology between class I and II MHC molecules was observed in an analysis of the *human* HLA-A2 class I and *murine* I-A^k class II molecules (8). This observation suggests that not only are class I and II molecules structurally similar but also that the homology may be conserved across species. To test if individual peptides can be immunogenic in both humans and rodents, two class I-restricted peptides analyzed in human immune responses, influenza A nucleoprotein 335-49 (NP335-49, reference 9) restricted by HLA-B37 and influenza matrix 55-73 (MA55-73, reference 10) restricted by HLA-A2, were analyzed for murine class II responses in CBA, BALB/c, and C57/B6 mice. Fig. 3 shows that both peptides generated a positive murine proliferative response, and in both cases the responding T cells were of the CD4$^+$ subset. For each peptide only one of the three haplotypes tested demonstrated a proliferative T cell response demonstrating the specificity of the peptide recognition (data from nonresponding haplotypes not shown). Thus, not only do peptides defined in murine class I-restricted systems function in murine class II systems, but also peptides defined in human class I-restricted systems function in murine class II-restricted systems.

To determine the class II MHC restriction, H-2 recombinant mice (B10.A(4R), B10.A(5R), B10.GD, and A.TL) were immunized with all seven peptides and lymph node proliferation assayed (data not shown). Based on these results the MHC re-
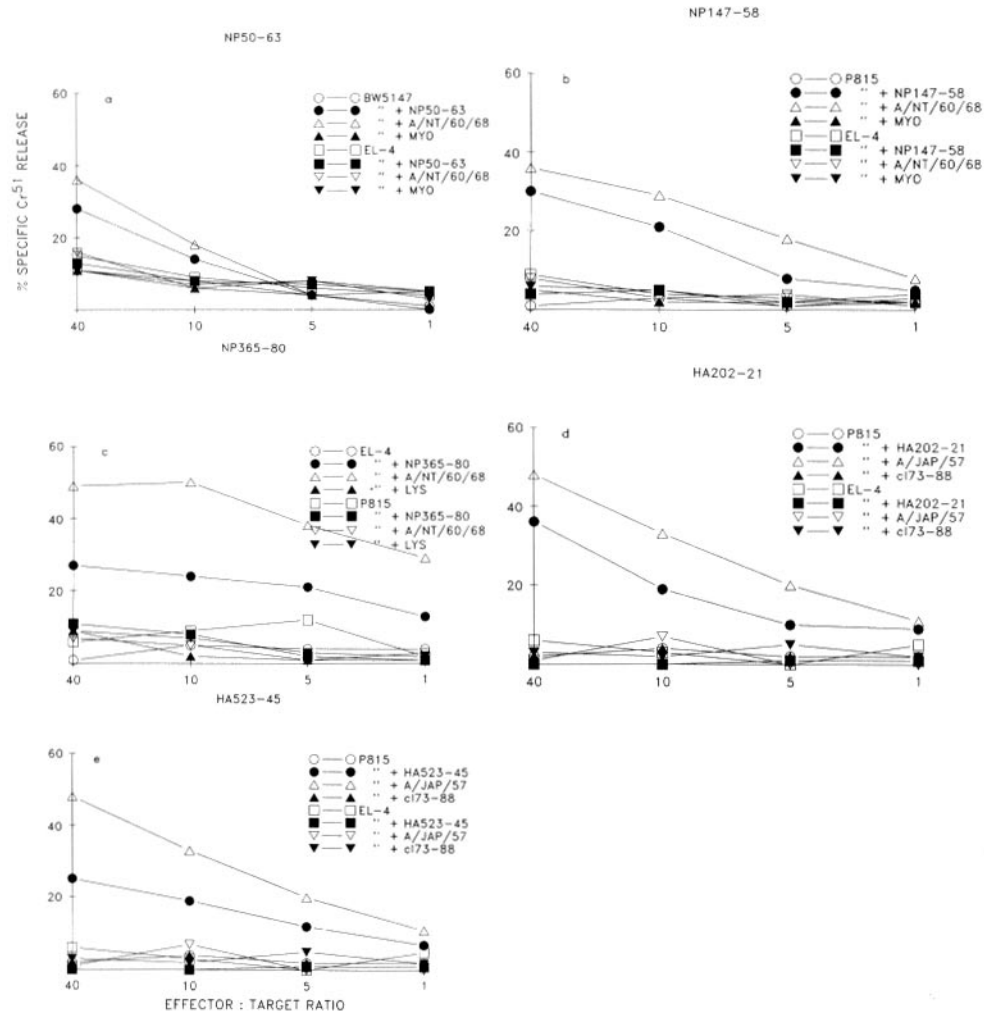
FIGURE 2.   Cytotoxicity analyzed by ^{51}Cr release using the same peptides as Fig. 1. Peptides are
(a) NP50-63, (b) NP147-58, (c) NP365-80, (d) HA202-21, (e) HA523-45. In each assay H-2-matched
target cells were infected with influenza A, or incubated with the indicated peptide. Control pep-
tides consisted of residues 132-146 of myoglobin (Myo), 46-61 of lysozyme (Lys), and 73-88 of
λ repressor cI (cI73-88). Syngeneic target cells (O), plus peptide (●), plus influenza A (Δ), and
plus control peptide (▲), and allogeneic target cells (□), plus peptide (■), plus influenza A (▽),
and plus control peptide (▼). Target cells were BW (a), P815 (b, d, and e), and EL-4 (c). Specific
Cr release was calculated as: (experimental − spontaneous counts)/(total − spontaneous counts).
Mouse strains were CBA (a), BALB/c (b, d, and e), and C57/B6 (c). Influenza A virus strains
were A/NT/60/68 (a, b, and c) and A/JAP/57 (d and e).

strictions are shown in Table III. Some peptides, e.g., NP50-63 and NP147-58, have
both a class I and II restriction functional in the same haplotype, H-2^k and H-2^d,
respectively. Other peptides, e.g., NP365-80 and HA523-45, demonstrate class I and
class II restriction only in different haplotypes (D^b vs. A^k and K^d vs. A^k, respec-
tively). One peptide, HA202-21, functions in three haplotypes in a class II–restricted
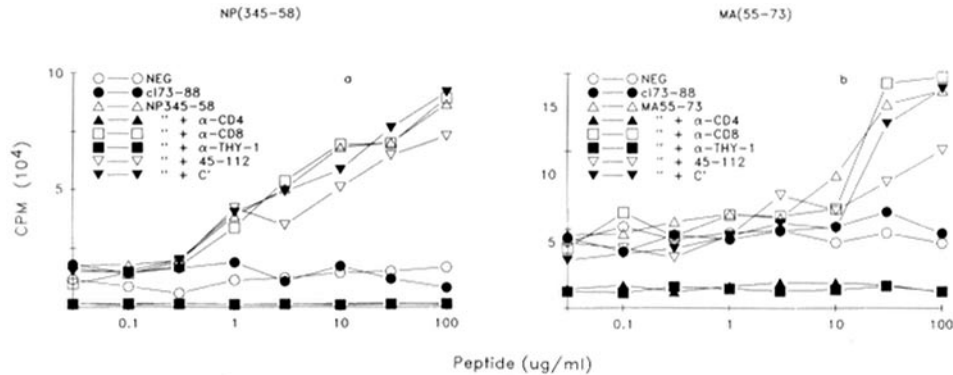
NP(345-58)   MA(55-73)

FIGURE 3. Murine lymph node proliferation analysis of peptides previously identified in human class I–restricted cytotoxic responses. Peptides are (a) NP335-49 and (b) MA55-73. Methods were the same as in Fig. 1. No peptide and no mAb or C' (○), cI73-88 (peptide 73-88 from λ repressor cI) with no mAb or C' (●), peptide and no mAb or C' (△), peptide plus anti-CD4 mAb (GK1.5) plus C' (▲), peptide plus anti-CD8 mAb (AD4) plus C' (□), peptide plus anti-Thy-1 mAb (13.4) plus C' (■), peptide plus anti-ARS mAb (45-112) plus C' (▽), peptide plus C' without mAb (▼). Mice were CBA (a) and BALB/c (b).

manner as well as class I K^d. Also shown are the crossreactivities between the human class I and murine class II restrictions, HLA-B37 with I-A^k and HLA-A2 with I-A^d. It is striking that all seven peptides use an I-A class II MHC restriction. However, based on the limited number of currently defined class I peptides available to analyze, it is not clear if the incidence of I-A restrictions is a coincidence or reflects some unknown structural feature. Experimenta to investigate if class II–restricted

TABLE III

Class I and Class II MHC Restrictions of Peptides

Peptide	Class I	Class II
Interclass Recognition		
Nucleoprotein		
NP50-63	K^k	A^k
NP147-58	K^d	A^d
NP365-80	D^d	A^k
Hemagglutinin		
HA202-21	K^d	$A^{b,d,k}$
HA523-45	K^d	A^k
Interspecies Recognition		
Nucleoprotein		
NP335-49	HLA-B37	A^k
Matrix		
MA55-73	HLA-A2	A^d

Peptides are amino acids 50-63 (NP50-63)117, 147-58 (NP147-58, reference 12), 335-49 (NP335-49, reference 9), and 365-80 (NP365-80, reference 9) from the nucleoprotein and 55-73 (MA55-73, reference 10) from the matrix protein of influenza A/NT/60/68, and 202-21 (HA202-21)139 and 523-45 (HA523-45)13 from the hemagglutinin of influenza A/Jap/57. In addition to the class II MHC restrictions listed, additional I-E^d restrictions for NP147-58, M55-73, and HA202-21 could not be excluded.

peptides are in general immunogenic in class I responses are technically more difficult. Using primary in vitro stimulation of naive spleen cells (24), no class I–restricted cytotoxic responses were detectable against seven class II peptides examined in these experiments. Interpretation of these results is not definitive because T cells induced by primary in vitro stimulation frequently recognize different epitopes than T cells induced with in vivo immunization (24). However, the possibility that the requirements for class I binding of peptides may be more stringent than for class II peptide binding can not be excluded.

Discussion

Our data demonstrate that identical immunogenic peptides can stimulate CD8$^+$ cytotoxic T cell responses in class I–restricted assays and CD4$^+$ helper T cell responses in class II–restricted assays. Previous work by other groups has indicated similarities between class I and II MHC molecules (6–8), as well as between TCR molecules from class I and II MHC-restricted T cells (1–5). In conjunction with our data that identical peptides demonstrate crossreactivities in both class I and II–restricted systems, all three molecules (TCR, MHC, and antigen) involved in T cell recognition of processed antigen in both class I and II, as well as human and murine restricted systems, are structurally and functionally similar. Previous reports of class II–specific CD8$^+$ T cells (although occurring at low frequency) are consistent with the idea of similar recognition mechanisms in the class I and II systems (25). This is further supported by the isolation of a T cell clone that specifically recognizes both a class I (D^b) and a class II (I-E^k) molecule (26). In the studies described here it is striking that in at least one of the three strains tested (H-2^{b,d,k}), class II responses were identified against all seven previously defined class I peptides.

The data also indicate that although mechanisms of T cell recognition of antigen are similar in both systems, the immune response to a specific immunization produces either a class I or a class II response to a particular peptide even in mouse strains potentially capable of responding via both types of MHC restriction. For example, CBA mice could potentially respond to NP50-63 via both class I K^k and class II A^k. However, depending upon the mode of immunization either a class I (after immunization with live virus) or class II (after immunization with peptide plus adjuvant) response predominates (Figs. 1 a and 2 a). One hypothesis consistent with this observation is that class I MHC molecules have more stringent requirements for peptide binding; therefore, class I–restricted peptides are a subset of all immunogenic peptides. Another, not mutually exclusive hypothesis is that processed antigens from intracytoplasmic proteins associate with class I molecules, and extracellular antigens from soluble proteins associate with class II molecules via two separate pathways of processing (27–28). Further support for this hypothesis is based on recent experiments demonstrating that APCs plus soluble ovalbumin induce class II T cell responses; whereas, if the protein is introduced intracytoplasmically by hypotonic vesicles, then class I responses are induced (24). Experiments are currently underway to test this hypothesis using class I and II–restricted T cells that recognize the identical peptide. In conclusion, the mechanism by which T cells recognize peptides or processed antigen in the context of MHC molecules appears to have been evolutionarily conserved by the class I and class II MHC systems and across species by humans and mice.

Summary

Previous data from many groups show that both class I and class II–restricted T cells recognize short synthetic peptides in the context of their respective MHC molecules (9–18). all of the peptides described to date are restricted to only a single class of MHC molecules; however, structural homology between the class I and II MHC molecules and the use of similar TCRs by class I and II–restricted T cells suggest that antigen recognition mechanisms are similar in both systems. To directly compare antigen recognition in the two systems, we analyzed peptides for the ability to function in both a class I and II–restricted system and found that seven of seven individual peptides tested stimulate both class I and II–restricted T cell responses. In addition, two of the peptides can function in different species stimulating both human class I and murine class II T cell responses. Thus, the process of T cell recognition of antigen in the context of MHC molecules was highly conserved in evolution not only between the class I and class II MHC systems, but also between the murine and human species.

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