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Detection of Antigen-Specific T Cells with Multivalent Soluble Class II MHC Covalent Peptide Complexes

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Summary

Multimeric soluble MHC class II molecules stably occupied with covalently attached peptides bind with appropriate specificity to T cell hybridomas and T cells from T cell receptor transgenic mice. There is a direct correlation between soluble T cell receptor affinity for monomeric MHC/peptide and level of binding of multimeric MHC/peptide to T cells. While binding of the multimeric MHC/peptide complex is proportional to T cell receptor affinity and expression level, there is little influence of T cell CD4.

Introduction

The ability of antibody to bind directly to antigen has been a useful tool in following the fate and migration pathway of antigen-specific B cells during the course of an immune response (Biozzi et al., 1967; Sercarz and Modabber, 1968; Greenstein et al., 1980) or establishment of self-tolerance (Goodnow et al., 1990). Antigen–antibody interactions have also been used to document the maturation of antibody affinity through primary and secondary immune responses (Berek and Milstein, 1987). Such experiments have been much harder with T cells since the ligand for the T cell α/β antigen receptor (αβTCR) is not a free antigen but the combination of an antigenic peptide fragment bound to a self-MHC molecule. As a substitute, a number of investigators have used monoclonal antibodies (MAbs) specific for the α/β combination of a particular αβTCR. Such anti-idiotypic reagents have been extremely useful in tracking particular T cells in vivo, especially when combined with mice bearing a transgenic αβTCR reactive with the MAb (Teh et al., 1989; Pape et al., 1997). However, this approach has required the sometimes difficult preparation of an individual anti-idiotypic MAb for each T cell clone to be studied and has not been useful in studying the normal heterogeneous T cell response to a particular MHC/peptide combination.

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Recently, methods for producing soluble MHC molecules and αβTCRs have been developed (Matsui et al., 1991; Matsumura et al., 1992; Stern and Wiley, 1992; Corr et al., 1994; Kappler et al., 1994; Kozono et al., 1994; Matsui et al., 1994; Garboczi et al., 1996; Garcia et al., 1996), allowing the measurement of the affinity of αβTCRs for MHC molecules loaded with particular peptides. These experiments have shown that these interactions are of very low affinity, with dissociation constants usually no better than 1–100 μM (Corr et al., 1994; Matsui et al., 1994; Alam et al., 1996; Fremont et al., 1996; Garcia et al., 1997; Khandekar et al., 1997; Seibel et al., 1997; Liu et al., 1998), too weak for these peptide-loaded MHC molecules to be useful directly in detecting peptide-specific T cells. However, recently several studies (Altman et al., 1996; McHeyzer-Williams et al., 1996; Murali-Krishna et al., 1998) have shown that when soluble MHC/peptide complexes are multimerized, they achieve much higher avidities for the αβTCR on the T cell surface, presumably via cooperative multivalent binding.

In the current study, we combined the use of soluble multivalent MHC class II molecules with a method that assures complete stable occupancy of the MHC binding groove with a single peptide by genetically attaching the peptide to the MHC molecule with a flexible linker. We prepared a variety of these reagents and show that they bind specifically to normal T cells and T cell hybridomas. We also show that the level of binding is proportional to the affinity of the αβTCR for monomeric MHC/peptide.

Results

Specific Binding of Multivalent MHC/Peptide to T Cell Hybridomas

As described in the Experimental Procedures, soluble IE^k bearing a covalently attached moth cytochrome c (MCC) or mouse hemoglobin (Hb) peptide was prepared with a biotinylated peptide tag on the β chain C terminus (bio-IE^kMCC and bio-IE^kHb). Purified multivalent complexes of these MHC molecules with phycoerythrin/streptavidin (PESA) were prepared. Various concentrations of the bio-IE^kMCC-PESA complex were incubated with a T cell hybridoma, KMAC-92, specific for IE^k/MCC, for 2 hr at 37°C in complete tissue culture medium containing 10% FCS and 5 mM NaN_3. As a negative control, the hybridoma cells were incubated identically with the bio-IE^kHb-PESA complex. The cells were then washed and the bound bio-IE^kpeptide-PESA complexes analyzed by flow cytometry. The results are shown in Figures 1A and 1B. Virtually no binding of the control Hb-containing complex was seen at any dose (Figure 1A), but the specific MCC-containing complex bound strongly in a dose-dependent manner (Figure 1B). Saturation binding was not seen even at 32 μg/ml of the complex; however, to conserve reagent most subsequent experiments were performed at 20 μg/ml.

The time course of binding of the bio-IE^kMCC-PESA complex was assessed, again using the Hb peptide-containing complex as the negative control. Cells were

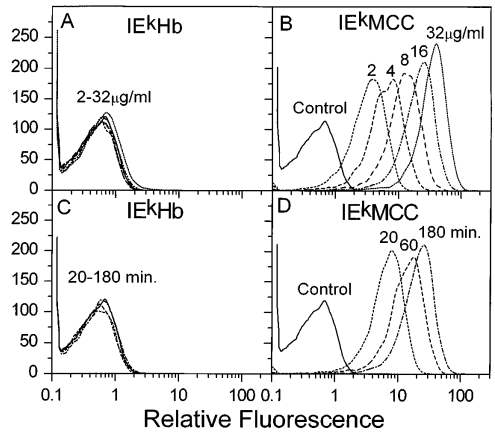

Figure 1. Specific Binding of Multivalent IE^{k}MCC to a T Cell Hybridoma

KMAC-92 hybridoma cells (1×10^5) were incubated with various concentrations of (A) bio-IE^{k}Hb–PESA or (B) bio-IE^{k}MCC–PESA in a volume of 50 μl of complete tissue culture medium containing 5 mM NaN_3 for 2 hr at 37°C. The cells were washed 3× with PBS containing 2% FCS and 5 mM NaN_3 and analyzed for bound PE fluorescence. Under similar conditions, KMAC-92 cells were incubated with 20 ug/ml (C) bio-IE^{k}Hb–PESA or (D) bio-IE^{k}MCC–PESA for various times before washing and flow cytometric analysis.

incubated with the reagents for various times at 37°C in complete tissue culture medium containing 10% FCS and 5 mM NaN_3 before they were washed and analyzed. The results are shown in Figures 1C and 1D. Again, no significant binding of the Hb-containing complex was seen at any time tested (Figure 1C). Strong binding of the MCC complex was seen within 20 min (Figure 1D). Roughly twice as much was bound by 1 hr and three times as much by 3 hr. In subsequent experiments, incubations with the complexes were for 2–3 hr.

Two additional versions of the bio-MHC class II peptide–PESA complexes were prepared as described in the Experimental Procedures. The first contained IE^k with a variant of the MCC peptide in which amino acid 99 was changed from lysine to alanine (IE^{k}MCC99A). The other contained IA^d with a peptide corresponding to amino acids 328–338 of chicken ovalbumin (IA^{d}OVA). The four multimeric reagents were used to stain a series of T cell hybridomas of various specificities. In each case, the hybridoma was incubated with a complex containing the appropriate MHC/peptide combination and, as a negative control, with a complex containing an inappropriate MHC/peptide combination. In addition, the hybridomas were incubated with an anti-Cβ monoclonal antibody to measure total surface αβTCR. The results are shown in Figure 2. Complexes containing the appropriate MHC/peptide combination bound to each T cell hybridoma, although the extent of binding varied over more than a 100-fold range. Poor to no binding was seen with the inappropriate combination, demonstrating the highly specific nature of the reagents.

Factors Influencing the Binding of Multivalent MHC/Peptide to T Cell Hybridomas

We performed a series of experiments to evaluate factors influencing the wide range of binding shown in Figure 2. We postulated that at least three factors could play a role in the amount of binding of the multivalent complexes: (1) the level of αβTCR expression; (2) the affinities of the αβTCRs for the MHC/peptide ligand; and (3) the relative involvement of CD4/class II MHC interactions in stabilizing the binding.

To test the relation between αβTCR levels and the level of binding, we prepared a series of subclones from the T cell hybridoma KMAC-92 with surface αβTCR levels that varied over a factor of three. The binding of bio-IE^{k}MCC–PESA to the clones was compared to that of

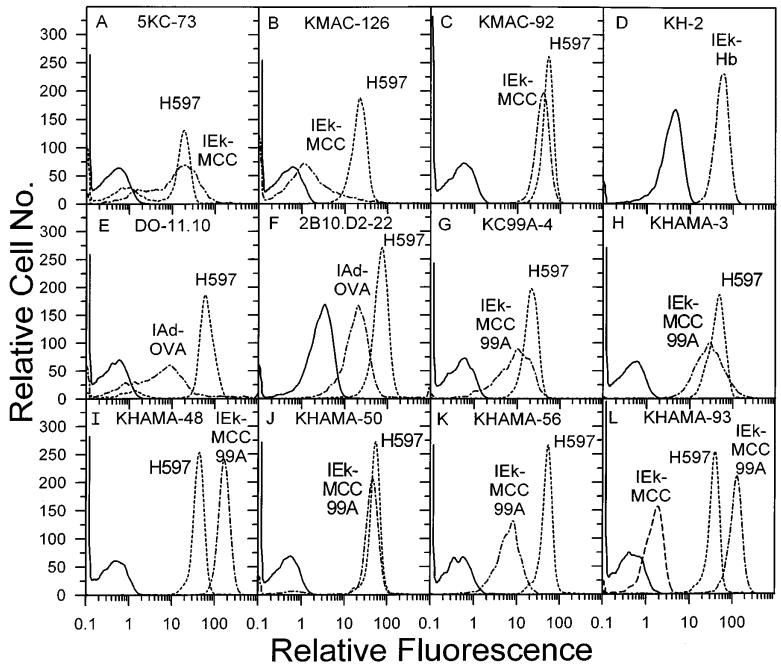

Figure 2. Summary of Binding Studies of Multivalent MHC/Peptide to T Cell Hybridomas

In each of the 12 panels, 1×10^5 hybridoma T cells of known MHC/peptide specificity were incubated for 2 to 3 hr with 20 μg/ml of PESA complexed with the appropriate bio-MHC/peptide. The cells were washed and analyzed for bound PESA (unevenly dashed line) as in Figure 1. Except for hybridoma KH-2 in panel D, aliquots of the cells were labeled also with the anti-mouse Cβ antibody, H597 (dotted line). As a negative control, cells were prepared identically with complexes containing an inappropriate peptide and/or MHC molecule (solid line). This negative reagent was bio-IE^{k}Hb–PESA in panels A, B, C, H–L, and bio-IE^{k}MCC–PESA in panels D–G. Finally, in panel L, in addition to the bio-IE^{k}MCC99A–PESA, the KHAMA-93 hybridoma cells were incubated with bio-IE^{k}MCC–PESA (dashed line).

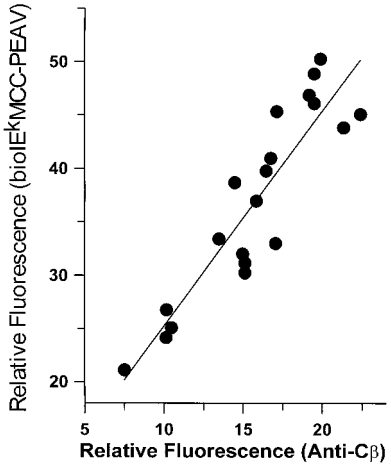

Figure 3. Correlation between Level of Surface αβTCR and Binding of Multivalent MHC/Peptide to T Cell Hybridomas

The hybridoma KMAC-92 was cloned at limiting dilution and 20 clones were assessed for binding of either bio-IE^{k}MCC–PESA or an anti-Cβ MAb as in Figures 1 and 2.

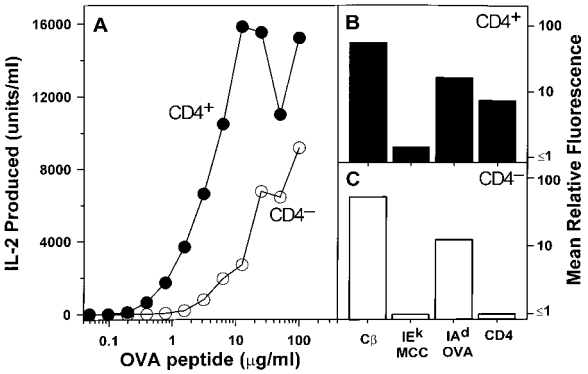

Figure 4. Lack of Influence of CD4 on Binding of Multivalent MHC/Peptide

(A) A CD4$^+$ (closed circles) and CD4$^-$ (open circles) clone of the hybridoma B10.D2–22 were tested for response to various concentrations of OVA327–339 peptide presented by the IA^d-bearing BALB/c lymphoma A20. The data are presented as units of IL-2 produced versus peptide concentration.

(B and C) The CD4$^+$ (closed bars) and CD4$^-$ (open bars) clones of B10.D2-22 were incubated with bio anti-Cβ + PESA, fluorescein anti-CD4, bio-IE^{k}MCC–PESA, or bio-IA^{d}OVA–PESA. The data are presented as the mean fluorescence of the labeled population corrected for the fluorescence seen with nothing (CD4) or PESA alone (others).

an anti-Cβ monoclonal antibody. The results are shown in Figure 3. Over this range of expression, there was a linear correlation between the level of αβTCR expression determined with the anti-Cβ reagent and the binding of the MHC/peptide complex.

Next, we examined the possible role of CD4 in the binding of these reagents to the T cell hybridomas. CD4 can enhance signaling through the αβTCR by binding to MHC class II and recruiting p56lck to the signaling complex (Veillette et al., 1988). It has been suggested that by interaction with both MHC class II and the αβTCR, CD4 can also enhance signaling via stabilization of the αβTCR/MHC complex (Doyle and Strominger, 1987; Gay et al., 1988; Portoles and Janeway, 1989). It was possible that the geometry of the multivalent class II could still allow for this interaction, increasing the stability of the complex bound to the T cell surface. To test this idea, we examined CD4$^+$ and CD4$^-$ subclones of a T cell hybridoma, 2B10.D2-22, specific for IA^d/OVA.

We established that the IL-2 response of this T cell hybridoma to an OVA peptide presented by an IA^d-bearing B cell line was dependent on CD4. The results are shown in Figure 4A. The CD4$^-$ subclone of the hybridoma required about a 10-fold greater concentration of the OVA peptide and produced a lower plateau response than the CD4$^+$ subclone, despite the fact that both had an equivalent level of surface αβTCR (Figures 4B and 4C). We also compared the binding of bio-IA^{d}OVA–PESA to each of the hybridoma subclones (Figures 4B and 4C). The reagent bound equally well to both subclones, although no significant binding of a negative reagent, bio-IE^{k}MCC–PESA, was seen. These results suggest that, at least under these conditions, CD4 does not contribute significantly in stabilizing the binding of these multivalent MHC/peptide reagents to the T cell surface.

Finally, we examined the relation between receptor affinity and level of binding of the multimeric MHC/peptide. We have previously used surface plasmon resonance to measure the affinities of soluble αβTCRs prepared from a number of the T cell hybridomas shown in Figure 2 for their MHC/peptide ligands (Seibel et al., 1997; Liu et al., 1998). To complement these measurements, we prepared soluble αβTCR from an additional T cell hybridoma, KHAMA-93, as described in the Experimental Procedures. This hybridoma bound its multivalent-specific ligand, IE^{k}MCC99A, as well as any hybridoma we have tested and showed very weak but detectable cross-reactive binding to the related multivalent ligand containing IE^{k}MCC (Figure 2L). As described in the Experimental Procedures, we used the BIAcore system to measure the affinities of this receptor for monovalent versions of these MHC/peptide ligands. The soluble αβTCR was immobilized in the flow cells of a biosensor chip. Various concentrations of monovalent IE^k bearing either peptide were injected and the binding kinetics followed.

Figure 5A shows the data for IE^{k}MCC99A. This reagent bound the KHAMA-93 αβTCR extremely well. The k_a was calculated as 36000 $M^{-1}s^{-1}$ and the k_d as 0.176 s^{-1} for a calculated overall K_D of 4.9 μM and a complex half-life of about 8 seconds. This is among the highest affinity αβTCRs described for MHC class II/peptide ligands. The maximum binding values in Figure 5A were used to construct a Scatchard plot (Figure 5C). The K_D of the interaction calculated from this equilibrium data was 4 μM, in good agreement with the kinetic data. The KHAMA αβTCR bound the IE^{k}MCC ligand very poorly (Figure 5B), in agreement with the binding data in Figure 2L. Barely detectable binding was seen only at very high MHC concentrations (15–60 μM). This binding was too weak to calculate a K_D directly from the binding kinetics; however, the maximal binding could be used to estimate a minimal K_D from the Scatchard plot in Figure 5C. If one assumes the same number of potential αβTCR binding sites in the flow cell for IE^{k}MCC as for IE^{k}MCC99A, one

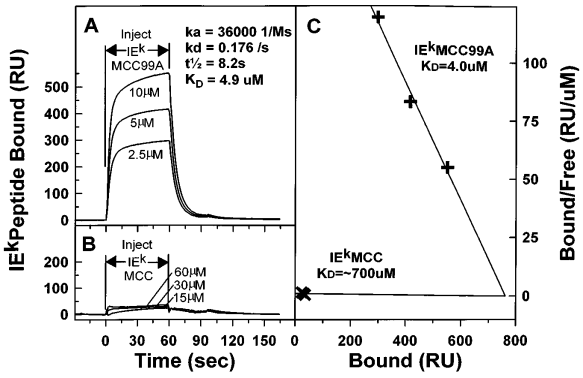

Figure 5. Affinities of the KHAMA αβTCR for Monomeric MHC Class II/Peptide

The binding kinetics of various concentrations of monomeric (A) IE^{k}MCC99A or (B) IE^{k}MCC to immobilized KHAMA-93 are shown. The class II molecules were injected at a flow rate of 10 ul/min for 1 min, and the binding curves have been corrected for bulk fluid phase signal. The IE^{k}MCC99A binding kinetics were analyzed with standard BIAcore software, and the average calculated k_a, k_d, half-life of the complex and K_D are shown. (C) The maximal binding data in panel A were used to construct a Scatchard plot for IE^{k}MCC99A binding to the immobilized KHAMA-93 αβTCR. A least squares regression line was fit to the data and the K_D of the interaction estimated from the negative reciprocal of the slope of the fitted line. The intersection of this line with the x-axis estimated the maximal binding capacity of the flow cells as 760 RU. By assuming the same potential binding capacity for the poorly binding IE^k-MCC ligand, the K_D of this interaction was approximated.

can estimate that the K_D of the IE^{k}MCC for the αβTCR is about 700 μM.

These results suggest a direct correlation between receptor affinity and the binding of the multivalent MHC/peptide complex to the T cell hybridoma. This point is made even more dramatically in Figure 6, which shows

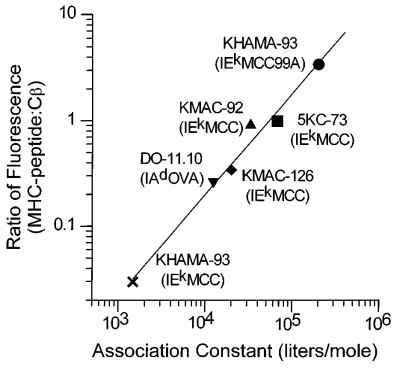

Figure 6. Correlation of Binding of Multivalent MHC/Peptide to T Cell Hybridomas with αβTCR Affinities

The binding of bio-MHC/peptide–PESA to T cell hybridomas averaged from several experiments and presented as the ratio of binding of the MHC reagents to binding with an anti-Cβ MAb are plotted versus the average affinity (K_A, liters/mole) of the corresponding soluble αβTCR for monovalent MHC-peptide. The MHC/αβTCR combinations were: (closed circle) IE^{k}MCC99A, KHAMA-93; (square) IE^{k}MCC, 5KC-73; (closed triangle) IE^{k}MCC, KMAC-92; (closed diamond) IE^{k}MCC, KMAC-126; (upside down closed triangle) Pooled data for IA^{d}OVA with DO-11.10 and 2B10. D2–22, since these hybridomas have identical receptors (Seibel et al., 1997); and (multiplication sign) IE^{k}MCC, KHAMA-93.

the relation between soluble αβTCR affinities for monovalent MHC/peptide and the binding of multivalent MHC/peptides for all of the T cell hybridomas for which we have been able to make both measurements. In this figure, the MHC/peptide binding has been normalized to the level of αβTCR surface expression on each hybridoma, and the affinities are reported as K_As rather than K_Ds. There is a striking linear relationship between the αβTCR affinities and the binding of the multimeric MHC/peptide to the T cell hybridoma over a 100- to 1000-fold range.

Binding to Normal and αβTCR Transgenic T Cells

These experiments established the ability of these multimeric class II MHC/peptide complexes to bind specifically to the αβTCR on T cell hybridomas. As a final experiment, we wished to establish that these reagents were capable of detecting the αβTCR on normal T cells. Therefore, we examined their binding to T cells taken from mice transgenic for Vα11/Vβ3 αβTCRs specific for IE^k/MCC. Two types of mice were used. One carried the AD10 and the other the AND αβTCR (Kaye et al., 1992). As a control, T cells from normal IE^k-bearing B10.BR mice were used. We examined these T cells for binding of bio-IE^{k}MCC–PESA, bio-IE^{k}MCC99A–PESA, and as a control, binding of an anti-Vβ3 monoclonal antibody. The results are shown in Figure 7.

Virtually no binding of either MHC reagent was seen to normal B10.BR CD4$^+$ T cells, an expected finding since the frequency of T cells reactive to these peptides should be very low in unimmunized mice (Figure 7A). On the other hand, bio-IE^{k}MCC–PESA bound very strongly to the majority of CD4$^+$ T cells from the transgenic mice (Figures 7B and 7C). Binding of bio-IE^{k}MCC99A–PESA was undetectable to CD4$^+$ T cells from the mice carrying the AD10 αβTCR and severely reduced compared to the MCC containing reagent with CD4$^+$ T cells from mice bearing the AND αβTCR. This was expected, since the lysine at position 99 of the MCC peptide has been shown to be critical for T cell recognition.

The level of Vβ3 on the surface of CD4$^+$ T cells from all three mice was similar (Figure 7D), indicating that the binding of the bio-IE^{k}MCC–PESA did not require an unusually high surface level of αβTCR. Virtually all CD4$^+$ T cells from both transgenic mice expressed high levels of the transgenic Vβ3-bearing β chain. However, only 70% of the AD10 T cells bound bio-IE^{k}MCC–PESA strongly (Figure 7D). Most of the remaining cells bound the reagent poorly, but significantly better than did the normal B10.BR control T cells. This most likely reflected the expression of a second α chain in these cells that variably suppressed, but did not eliminate, the expression of the transgenic α chain.

Discussion

Recent experiments with soluble αβTCRs and MHC molecules have revealed much about the nature of their interaction. Experiments with surface plasmon resonance have shown that αβTCRs have low affinities for their activating MHC/peptide ligands, with K_Ds typically in the range of 1–100 μM (Corr et al., 1994; Matsui et

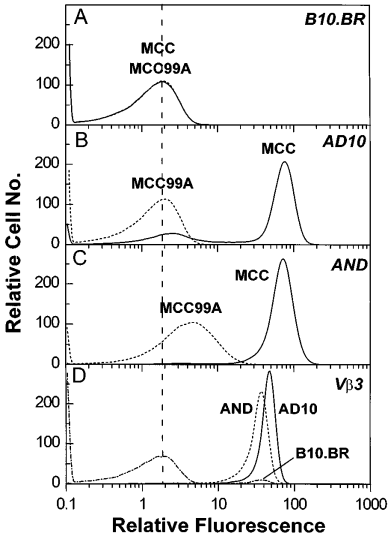

Figure 7. Detection of Normal T Cells Bearing a Transgenic αβTCR

Nylon fiber nonadherent T cells were purified from lymph nodes of normal (A) B10.BR mice or B10.BR mice expressing either the (B) AD10 or (C) AND transgenic Vα11/Vβ3-bearing αβTCR. Separate aliquots of the cells were incubated with fluorescein-labeled anti-CD4 and either bio-IE^{k}MCC–PESA (straight line) or bio-IE^{k}MCC99A–PESA (dotted line). Histograms show the PE fluorescence of the CD4$^+$ cells. (D) Aliquots of the three types of T cells were incubated with fluorescein-labeled anti-CD4 and biotinylated anti-Vβ3 (detected with PESA). The histograms show the PE fluorescence of the CD4$^+$ cells.

al., 1994; Alam et al., 1996; Fremont et al., 1996; Garcia et al., 1997; Khandekar et al., 1997; Seibel et al., 1997; Liu et al., 1998). MHC/peptide ligands involved in T cell positive and negative selection can have even lower affinities (Ashton-Rickardt and Tonegawa, 1994; Sebzda et al., 1994; Alam et al., 1996; Lyons et al., 1996; Liu et al., 1998). These affinities are too low to use monomeric soluble MHC molecules as reagents for detecting antigen-specific T cells. Recently, however, multimeric class I or class II MHC molecules have been shown to have increased avidity for T cells bearing the αβTCRs of appropriate specificity (Altman et al., 1996; McHeyzer-Williams et al., 1996; Murali-Krishna et al., 1998), presumably because of the cooperative effect of multipoint binding.

A critical requirement of such reagents is the complete occupancy of the MHC molecule with a single peptide, since heterogeneity in the multimer reduces the potential for multipoint binding. MHC molecules that are empty or poorly loaded have been prepared in various expression systems and subsequently loaded uniformly in vitro with a single peptide (Matsui et al., 1991; Matsumura et al., 1992; Stern and Wiley, 1992; Altman et al., 1993; Kozono et al., 1994). Because the stability of empty MHC molecules is isotype and allele specific, the efficiency of this in vitro peptide loading approach is quite variable. We have largely solved this problem for MHC class II by genetically coupling the peptide of interest to the N terminus of the β chain via a flexible linker, so that the peptide is covalently attached to the MHC molecule and stably occupies the peptide binding groove during biosynthesis (Kozono et al., 1994, 1995). In this paper, we show that the combination of a covalently attached peptide and multimerization via a biotinylated C-terminal tag produces a reagent that binds to T cells and T cell hybridomas with appropriate specificity.

In addition to improving peptide homogeneity, the peptide linker should improve the long-term stability of these reagents. Although dissociation of peptides from class II is generally very slow, it is significant over the course of many days, while we have found peptide occupancy with covalently bound peptides is stable over many months. One potential problem with this covalent construction is that occasionally the linker attached to the peptide C terminus may get in the way of αβTCR binding. In the one case where we have seen this interference, recognition was restored by proteolytic cleavage of the linker after assembly (data not shown). This cleavage is most easily accomplished by including a thrombin recognition sequence within the linker (Kozono et al., 1994).

These types of reagents have obvious usefulness in identifying and tracking antigen-specific T cells during normal or pathogenic immune responses. In addition, our results show that the level of binding of multimeric MHC class II/peptide complexes to T cells is directly proportional to the affinity of the αβTCR for the MHC class II/peptide complex. The need to know αβTCR affinities has become important in understanding the function of T cells. For example, differences in the affinities of αβTCRs for positively selecting versus deleting or activating MHC ligands has been hypothesized to account for the repertoire of T cells that make up the mature peripheral repertoire (Ashton-Rickardt and Tonegawa, 1994; Hogquist et al., 1994; Sebzda et al., 1994; Alam et al., 1996; Lyons et al., 1996; Liu et al., 1998). In the periphery, the relative affinity of T cells for an MHC ligand may play a part in the decision between productive activation versus anergy or deletion (Matsui et al., 1994; Alam et al., 1996; Lyons et al., 1996). Thus, αβTCR affinity may play a role in the escape of autoimmune T cells from negative selection. Methods measuring the affinities of αβTCRs for their MHC/peptide ligands have improved considerably in recent years; however, they require the sometimes difficult task of producing high quantities of native soluble αβTCRs. In addition, measurements of affinities in the range of K_As between 10^3 and 10^4 M^{-1} are extremely costly and difficult. Our results suggest that αβTCR affinities can be estimated in the range of about 10^3 to 10^6 M^{-1} with these multimeric reagents, making them very useful in addressing some of these questions.

With the protocols we used here, we were unable to detect any contribution of CD4 to the overall level of binding with these reagents. Since the ability of CD4 to interact with class II MHC is well documented (Doyle and Strominger, 1987; Gay et al., 1988), either the geometry of these reagents precludes incorporation of CD4 or the interaction is of such low affinity that it does not contribute significantly to the overall binding avidity at this valency.

Experimental Procedures

Soluble MHC Class II Molecules with Covalent Peptides

Soluble MHC class II molecules were prepared using baculovirus as previously described (Kozono et al., 1994, 1995). The IE^k molecule

was produced in combination with three different peptides: a peptide corresponding to amino acids 88–103 of moth cytochrome c (Schwartz et al., 1985); this same peptide with the lysine at position 99 changed to alanine (MCC99A) (Reay et al., 1994); and a peptide corresponding to amino acids 64–76 of mouse β-hemoglobin (Evavold et al., 1992). The IA^d molecule was produced bound to a peptide corresponding to amino acids 328–338 of chicken ovalbumin (OVA) (Shimonkevitz et al., 1984). In addition to those previously reported, new constructions were prepared by adding a sequence to the 3′ end of each MHC β chain encoding a peptide capable of being biotinylated by the *Escherichia coli* enzyme BirA (Schatz, 1993). The final sequence was:

```
-E^k or A^d β2 ----><----Linker ----
ACGGTCGAGTGGAAAGCACAGTCCACTAGGGGT
 T  V  E  W  K  A  Q  S  T  R  G

----Linker -----><- Peptide Tag ---
GGAGCTAGCGGCGGTGGACTTGGAGGAATCTTT
 G  A  S  G  G  G  L  G  G  I  F

                                    SphI
------Peptide Tag ------>            \/
GAGGCAATGAAGATGGAGCTGCGGGACTGAGCATGCGGAT
 E  A  M  K  M  E  L  R  D  *
          /\
 Site of Biotin Addition
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Soluble MHC class II molecules were purified from culture supernatants of infected High Five (InVitrogen) or SF9 insect cells by immunoaffinity purification using monoclonal antibodies M5/114 (for IA^d) and 14-4-4 (for IE^k). The final sample was stored in phosphate-buffered saline with 5 mM NaN3, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.1 mM PMSF.

Biotinylated Soluble MHC/Peptide

Purified MHC class II molecules carrying the peptide tag were biotinylated (bio) using the enzyme BirA (Avidity, Denver, CO). Typically, 1 mg of protein at 1.8 mg/ml was incubated with 8–10 μg of BirA at 25°C for 16–20 hr in a buffer containing 10 mM Tris (pH 8), 50 mM Bicine (pH 8.3), 10 mM ATP, 10 mM Mg acetate, and 40 uM biotin. Following the reaction, the unbound biotin was removed and the buffer exchanged to PBS with 5 μM NaN_3 using Centricon-30 concentrators (Amicon, Beverley, MA). The extent of biotinylation was estimated by an ELISA of the soluble MHC class II before and after removal of biotinylated protein with avidin–agarose beads (Vector Labs, Burlingame, CA). Typically, ~85% of the protein was biotinylated.

Preparation of Fluorescent Soluble Multivalent MHC/Peptide Complexes

Fluorescent multivalent MHC/peptide complexes were prepared by incubating purified bio-MHC/peptide with a covalent phycoerythrin(PE)/streptavidin(AV) complex (BioSource International, Camarillo, CA) at a calculated molar ratio of bio-MHC/peptide to SA of 8:1. The mixture was then subjected to size exclusion FPLC chromatography on Superdex-200 to separate the large molecular weight bio-MHC/peptide–PESA complex from excess free MHC/peptide. The amount of uncomplexed bio-MHC/peptide recovered and SDS-PAGE analysis of the complex indicated that the four binding sites of SA for biotin were occupied with bio-MHC/peptide. However, since the ratio of SA to PE in the commercial reagent was 2:1, some portion of the complex may have had a valence higher than four, and/or some free SA bound to bio-MHC/peptide may have been present.

T Cell Hybridomas

The T cell hybridomas used in this study were produced as previously described (White et al., 1989). Most have been described in previous publications. Their specificities and names are as follows: IE^k/MCC-specific, 5KC-73 (White et al., 1993), KMAC-92 (Liu et al., 1997), KMAC-126 (Liu et al., 1997); IE^k/Hb-specific, KH-2 (not previously reported); IA^d/OVA-specific, D0.11.10 (White et al., 1983), 2B10.D2–22 (Seibel et al., 1997); IE^k/MCC99A-specific, KC99A-4 (Liu et al., 1997), KHAMA-3, KHAMA-48, KHAMA-50, KHAMA-56, and KHAMA-93 (not previously reported). The IA^d/OVA-specific αβTCRs on D0.11.10 and 2B10.D2-22 were of identical sequence (Seibel et al., 1997).

Mice

Mice carrying either the AD10 or AND transgenic Vα11/Vβ3 αβTCR specific for IE^k/MCC were a generous gift of Dr. Steven Hedrick, University of California, San Diego (Kaye et al., 1992). They were extensively backcrossed to $H\text{-}2^k$-bearing B10.BR mice. Normal B10.BR mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

Flowcytometric Analysis

The anti-Cβ MAb H597 (Kubo et al., 1989) was used to estimate surface αβTCR on T cell hybridomas, and the anti-Vβ3 antibody KJ25 (Pharmingen, La Jolla, CA) was used to detect Vβ3-bearing T cells. The anti-Cα MAb ADO-304 (Liu et al., 1998) was used to capture soluble αβTCR in biosensor flow cells. CD4 was detected with the MAb GK-1.5 (Dialynas et al., 1983). Cells labeled with fluorescent antibodies or soluble bio-MHC/peptide–PESA complexes were analyzed using an Epics XL flow cytometer (Coulter, Miami, FL).

Soluble αβTCR

The genes for the variable portions of the α and β chains of the T cell hybridoma KHAMA-93 were synthesized by PCR using cDNA prepared from the hybridoma. The sequences of the CDR3 regions of these gene segments were:

```
KHAMA-93 Alpha
TGTGCTGCCTCTCGGAATAGCAATAACAGA/
 C  A  A  S  R  N  S  N  N  R /
--AV11S2--->N<----------------/

                              AccIII
/GTGGTGAAGCCCAACATCCAGAATCCGGAA
/  V  V  K  P  N  I  Q  N  P  E
/---------><------------Cα---

KHAMA-93 Beta
TGTGCCTGGAGTAGACACCTTAACCAAGAC/
 C  A  W  S  R  H  L  N  Q  D /
--BV14S1--><---N--><--------/

                               BglII
/GGCACTCGGCTCCTCGTGTTAGAAGATCTG
/ G  T  R  L  L  V  L  E  D  L
/-------------------><---Cβ2--
```

The gene segments were cloned in-frame with the mouse Cα or Cβ genes in a baculovirus transfer vector and introduced by recombination into baculovirus as previously described (Kappler et al., 1994; Seibel et al., 1997; Liu et al., 1998). Soluble αβTCR was purified from the supernatants of infected High Five or SF9 insect cells as previously described (Kappler et al., 1994).

Surface Plasmon Resonance Studies

The BIAcore system (Biacore Inc., Piscataway, NJ) was used to evaluate the interaction of soluble MHC/peptide with soluble αβTCR as previously described (Seibel et al., 1997; Liu et al., 1998). In brief, purified soluble TCR was immobilized in a flow cell of a biosensor chip by capture with a high-affinity anti-Cα antibody. Various concentrations of the appropriate soluble MHC/peptide were injected

through the flow cell and the binding kinetics recorded. As controls, all preparations were injected through flow cells in which inappropriate αβTCR had been captured to correct for bulk fluid phase refractive index and any possible nonspecific binding. These data were subtracted from the experimental data. Standard BIAevalution software was used to analyze the kinetic data.

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