could not internalize E-IV.3 Fab. Greater truncation of huFc$_\gamma$RIIA abolished all phagocytic function. This differentiation between huFc$_\gamma$RII-mediated internalization of small complexes and large particles may explain some of the conflicting reports concerning the requirement of an $[Ca^{2+}]_i$ flux for huFc$_\gamma$RII-mediated phagocytosis in macrophages (*3, 21*). Apparently, the cytoplasmic tail of huFc$_\gamma$RIIA contains distinct functional regions for initiating the internalization of small complexes versus an $[Ca^{2+}]_i$ flux and the internalization of large particles. Thus, multiple signaling molecules may interact with the cytoplasmic domain. Bifurcation of the signal pathways utilized by huFc$_\gamma$RIIA to activate multiple effectors begins at the level of the receptor.

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Low Affinity Interaction of Peptide-MHC Complexes with T Cell Receptors

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The interaction of antigen-specific T cell receptors (TCRs) with their ligands, peptides bound to molecules of the major histocompatibility complex (MHC), is central to most immune responses, yet little is known about its chemical characteristics. The binding to T cells of a labeled monoclonal antibody to the TCR was inhibited by soluble class II MHC heterodimers complexed to different peptides. Inhibition was both peptide- and TCR-specific and of low affinity, with a $K_D = 4 \times 10^{-5}$ to 6×10^{-5} M, orders of magnitude weaker than comparable antibody-antigen interactions. This finding is consistent with the scanning nature of T cell recognition and suggests that antigen-independent adhesion precedes TCR engagement.

THE T CELL RECEPTOR POLYPEPtides occur as either αβ or γδ heterodimers in close association with the monomorphic CD3 polypeptides on the surface of T cells (*1*). Similar to antibodies, T cell receptor genes consist of multiple V-, J-, and D-like gene segments that join together in various combinations to endow individual T cells with unique specificities (*1*). Analyses of TCR sequences suggest that their protein structure is similar to that of immunoglobulin Fab' fragments (*2*). Despite these similarities, however, the integral membrane character of both TCRs and their ligands (*3*) may necessitate very different characteristics of interaction versus antibody-antigen recognition. In addition, self peptide–MHC complexes are also important for TCR selection in the thymus (*4*). To study these interactions better, we have recently expressed both a TCR heterodimer (*5*) and a class II MHC heterodimer, E^k (*6*) in a glycolipid-anchored (GPI) form, allowing ready solubilization by enzymatic cleavage. Cells bearing the GPI-linked E^k can present peptide antigens to most T cell hybridomas with a fine specificity that is indistinguishable from cells expressing wild-type molecules (*6*). In addition, the cleaved, soluble E^k protein can efficiently bind antigenic peptides and stimulate T cells of the appropriate specificity when bound to a plate (*6*).

We have approached the question of TCR affinity for its ligand by measuring the ability of soluble peptide-E^k complexes to compete with a monoclonal antibody (MAb) to the TCR. The formula of Cheng and Prusoff (*7*)

$$K_D^A = \frac{IC_{50}}{1 + ([B]/K_D^B)}$$

allows one to derive the K_D of a competitor, A, for its receptor using a ligand of known K_D and a determination of what concentration of A results in a 50% inhibition (IC_{50}) of B. In this case, the unknown is peptide-MHC and the reference ligand is the MAb KJ25 (*8*) whose binding is ablated by mutations in the CDR2 region of $V_\beta 3$, mutations that also disrupt T cell recognition (*9*). We used the T cell line 5C.C7 (*10*), which recognizes moth cytochrome c (MCC) and the hybridoma 228.5 (*11*), which is specific for a Lys^{99} to Glu^{99} variant, MCC(99E) (*12*). This hybridoma expresses a VDJ_β sequence identical to that of 5C.C7, and its α chain differs only in the V-J junctional re-

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gion and J region sequences (*11*).

To determine the affinity of the KJ25 MAb, we first made Fab' fragments by protease digestion, labeled them with ^{125}I (*13*) and performed saturation binding studies (*14*) to 5C.C7 cells (Fig. 1). We derived a $K_D = 2.4 \times 10^{-9}$ M, typical of many antibodies that recognize cell surface proteins (*15*) and a receptor number of 1×10^5 per cell, somewhat higher than reports in the literature [2.5×10^4 to 5.0×10^4 TCRs per cell (*16*)]. The MCC and MCC(99E) peptides were complexed to soluble E^k by incubation at low pH (5.0) for 3 days at 37°C and size excluded to remove aggregates and free peptide on a Sephadex G-100 column (Fig. 2). This procedure resulted in complexes that were 85 to 100% pure as determined by NH_2-terminal amino acid sequencing (*17*). To compare the abilities of these peptide-MHC complexes to stimulate the relevant T cells, we coated Immulon IV plates with the different preparations, added T cells, and measured the resulting lymphokine release (Fig. 2). The 5C.C7 T cells produced interleukin-3 (IL-3) in response to the MCC-E^k complexes but not to the MCC(99E)-E^k complexes. In contrast, 228.5 T cells produced IL-2 when stimulated by the MCC(99E)-E^k complexes, but did not respond to MCC-E^k complexes. Peptides presented by an E^k-bearing B cell lymphoma line were also included as a control. Thus the two TCRs, although similar in their sequences, were specific with respect to the peptides described above, whether complexed to soluble or cell-bound E^k.

We examined the ability of these peptide-E^k complexes to compete for labeled KJ25 Fab'-binding to native TCR-bearing T cells (Fig. 3). The MCC-E^k complexes competed with the KJ25 tracer for binding to 5C.C7 cells, whereas the MCC(99E)-E^k complexes did not. The extent of this inhibition reached 80% at 300-μM complex [21.0 mg/ml (*18*)], yielding an IC_{50} of 7.5×10^{-5} M. With the use of the Cheng-Prusoff formula discussed above, the K_D of MCC-E^k is 4.9×10^{-5} M. In contrast, on 228.5 cells the MCC(99E)-E^k complex specifically inhibited KJ25 binding IC_{50} of 8.0×10^{-5} M and K_D of 5.3×10^{-5} M and MCC-E^k did not (Fig. 3B). A single experiment with a third T cell, the MCC-specific hybridoma 2B4 (*19*), also produced a K_D of 5.3×10^{-5} M (*20*). Uncomplexed E^k had no inhibitory effect (*20*). KJ25 binding reaches equilibrium after 1 hour (20°C) and these numbers also represent equilibrium values for peptide-MHC, because a 1-hour incubation with peptide-MHC complexes before KJ25 addition has no effect on the inhibition curve (*20*). These calculations assume that the peptide-MHC complexes are 100% pure, if they were 85% pure, for example (the lower range indicated by sequencing), the K_Ds would be proportionately lower (4.2×10^{-5} to 4.5×10^{-5} M). Most or all of the peptide-MHC complexes are biologically active, because ≥80% of the molecules in such preparations are recognized by MCC-E^k–specific MAbs that also inhibit the appropriate T cell responses (*21*).

One potential problem with the work described above is that because the KJ25 binding site on the TCR (CDR2 of $V_\beta 3$) only partially overlaps with the peptide-MHC binding site (presumably all CDRs), the kinetics of inhibition may be affected. This is unlikely, however, as the peptide-MHC complexes have only to sterically hinder KJ25 binding to the T cell receptors in this type of assay. The degree of occupancy should therefore directly reflect the binding energy of the TCR–peptide-MHC interaction.

We found no major differences between $CD4^+$ T cells (5C.C7) and $CD4^-$ cells (228.5 and 2B4.11). It therefore seems likely that CD4 does not contribute significantly to the binding energy of TCR recognition per se as postulated in the coreceptor model (*22*), but that CD4 comigration with the TCR during recognition may serve more of a signaling function (*23*).

We conclude that the affinity of an individual TCR for its ligand is very low, in the range of 4×10^{-5} to 6×10^{-5} M. Although our data deals only with very similar T cell receptor heterodimers and peptides bound to a single MHC molecule, cytochrome c–reactive T cells seem typical of most antigen-specific helper T cell systems and thus the numbers derived above may be

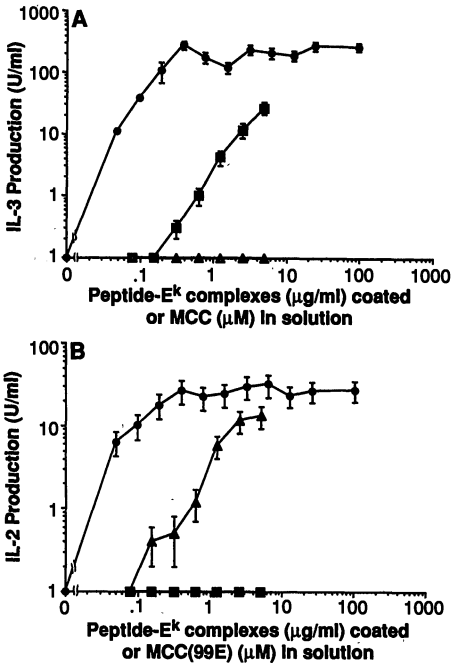

Fig. 2. T cell activation by peptide-MHC complex–coated plates. Soluble E^k heterodimers were purified from cells which express a GPI-linked chimeric E^k molecule using PI-PLC cleavage and antibody affinity chromatography as previously described (*6*). Soluble E^k at 720 nM was incubated with a 30-fold molar excess of peptide [MCC or MCC(99E)] in a citrate-phosphate buffer (pH 5.0) for 3 days at 37°C (*6, 36*). Volume was reduced using Centricon-30 (Amicon) concentrators, and the complexes were then applied to a Sephadex G-100 column equilibrated in PBS (pH 7.3). Fractions containing unaggregated protein (260/280 of <0.7) were pooled and reconcentrated. NH_2-terminal amino acid sequence analysis indicated that complexes prepared in this manner are 85 to 100% pure with respect to peptide (*17*). Microtiter plates (Immulon IV Dynatech Laboratories) were coated overnight at 4°C with either MCC-E^k or MCC(99E)-E^k complexes at the concentrations indicated. MCC-CH27 and MCC(99E)-CH27 refer to peptide in solution presented by the CH27 B cell lymphoma. Stimulation of 5C.C7 (**A**) or 228.5 (**B**) cells was measured by assaying either IL-3 or IL-2 production, respectively, as described (*6*). (A) IL-3 production of 5C.C7 cells stimulated with MCC-E^k (■), MCC(99E)-E^k (▲), or MCC-CH27 (●). (B) IL-2 production of 228.5 cells stimulated with MCC-E^k (■), MCC(99E)-E^k (▲), or MCC(99E)-CH27 (●).

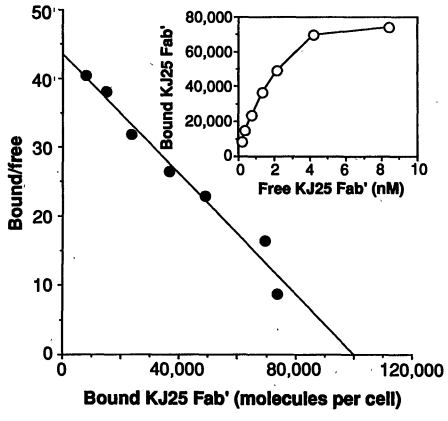

Fig. 1. Scatchard plot analysis of Fab' binding to 5C.C7 cells. KJ25 Fab' fragments were prepared (*33*) involving a pepsin digestion followed by size exclusion of $F(ab')_2$ fragments on G-100 Sephadex and reduction and alkylation to Fab' monomers. These monomers were purified by an additional size exclusion on a TSK 3000 SW column and labeled with ^{125}I (*13*). The specific activity of the ^{125}I-KJ25 Fab' was 6,300 cpm/ng as determined by self-displacement analysis and correcting for maximum binding ability (*34*). Binding assays were done as described (*35*). Briefly, serial dilutions of ^{125}I-KJ25 Fab' were incubated with 5×10^4 cells for 60 min at room temperature in a final volume of 15 μl of RPMI 1640 with 5% FCS. Cell-bound radioactivity was then separated from free material by centrifugation through a layer of fetal bovine serum. Nonspecific binding was defined as the cpm bound in the presence of a 200-fold excess of unlabeled KJ25 Fab'. The dissociation constant (K_D) and number of binding sites were determined by Scatchard analysis (*14*). Saturation was confirmed by determining the K_D at several different cell dilutions.

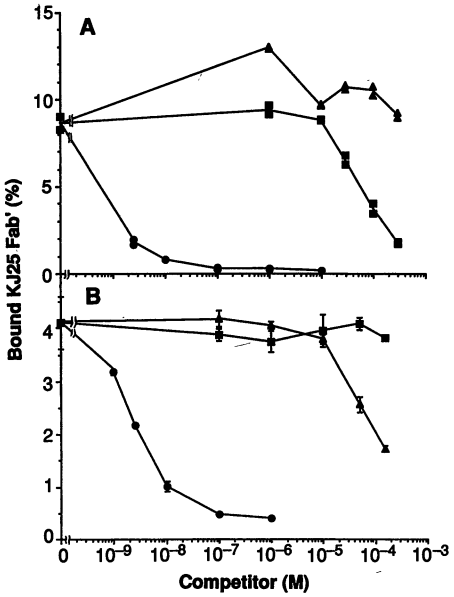

Fig. 3. Inhibitory effect of the MCC-E^k complexes to T cells. (**A**) The percentage of KJ25 Fab′ binding to 5C.C7 cells inhibited by unlabeled KJ25 Fab′ (●), MCC-E^k (■), or MCC(99E)-E^k (▲) plotted as duplicate points for each curve. (**B**) KJ25 Fab′ binding to 228.5 cells as inhibited by unlabeled KJ25 Fab′ (●), MCC-E^k (■), or MCC(99E)-E^k (▲) plotted as the mean plus the standard deviation (error bars) in triplicate. The 5C.C7 T cells (2.5×10^4) or 228.5 T cells (2.5×10^5) were incubated with 1.25 nM ^{125}I-KJ25 Fab′ and various concentrations of unlabeled KJ25 Fab′, MCC-E^k complexes, or MCC(99E)-E^k complexes for 1 hour at room temperature. After this incubation, cells were spun through a layer of FCS as in Fig. 1. The percent bound is defined as 100 × (cell-bound radioactivity/total). The concentration of receptor in the assay was $\approx 0.05\ K_D$. The error associated with depletion of the cold ligand (peptide-MHC) is not applicable in this case (*37*) as the affinity of the Fab′ is much higher. Five separate experiments showing 5C.C7 inhibition with MCC-E^k and two separate experiments showing 228.5 inhibition with MCC(99E) gave similar results (within a factor of 2).

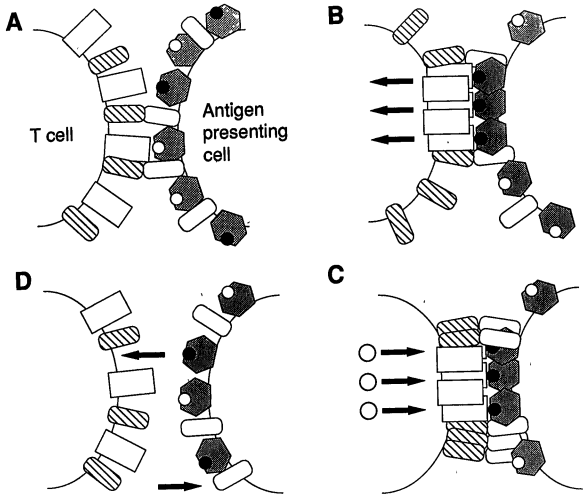

Fig. 4. Proposed steps in T cell activation. A random collision of a T cell with a potential target or antigen-presenting cell might first employ antigen-independent adhesion molecules (oblong shapes) to promote conjugate formation (**A**). This then produces a transiently stable interface between the cells which would allow TCR-CD3 complexes (rectangles) on the T cell to survey various peptide-MHC complexes (hexagons) on the presenting cell. Because of membrane fluidity and the large excess of TCRs, the longer the cell-cell conjugate exists the more MHC molecules could be surveyed, allowing MHC molecules with an appropriate peptide (filled circles) to congregate at the interface as in (**B**). This in turn would concentrate more TCRs at the interface and this density of TCRs and their colocalizing kinases and substrates could trigger T cell activation (arrows) once appropriate thresholds were reached. Because of the large excess of TCRs versus appropriate peptide-MHC complexes (1000 to 1, when antigen is limiting) and the very high K_Ds measured here, there may be many more TCRs at the interface than peptide-MHC molecules. If insufficient matches were found in the time the cells were bound together they would detach as in (D). In the event of TCR-dependent activation, however, the T cell would promote further adhesion as in (**C**) and reorganize cytoskeletally (*23*) to activate mechanisms that directionally secrete lymphokines or cytotoxic substances (arrows), depending on T cell type. Soon afterward, the enhanced adhesiveness declines and the cells may detach as in (**D**).

general. These values differ significantly from those reported for a cytotoxic T cell response that was specifically inhibited with a soluble form of a class I MHC molecule, with an apparent K_D of 10^{-7} M (*24*). Inhibition of biological function is often a complex matter, however, and the numbers obtained may only indirectly relate to the physical association of TCR with its ligand. Thus, our competition studies provide an estimate for this type of recognition and place TCRs below the range of typical hapten-specific antibodies (10^{-5} to 10^{-7} M) and even further from antibodies that bind large protein surfaces (10^{-8} to 10^{-10} M) (*15*), probably the most appropriate comparison (*25*). Although TCR affinity is many orders of magnitude less than analogous antibody-antigen interactions, the specificity is comparable, in that even very conservative changes in peptide or MHC residues can ablate recognition (*25, 26*). This presumably relates to the "scanning" mechanism by which T cells read MHC molecules on the surfaces of cells for potential ligands and then move on to survey other cells. This is in contrast to B lymphocytes, whose surface immunoglobulins must in many cases pull antigens out of solution and internalize them or secrete high affinity antibodies designed to bind antigenic surfaces with relatively long half-lives.

In view of the low affinities and relatively small numbers (50 to 200 per cell) of peptide-MHC complexes on presenting cells that can activate T cells (*27*), it seems unlikely that the initial event in T cell recognition could be TCR binding to peptide-MHC, because only a small number of MHC molecules with the appropriate peptide would be present in a given area of cell-cell contact. The resulting TCR–peptide-MHC complexes would dissociate almost as soon as they are formed. This suggests that cell-cell contact is initiated by adhesion molecules, consistent with observations that T cells can form conjugates with inappropriate presenting cells (*23, 28*) as proposed by Spits *et al.* (*29*).

How this might lead to TCR-mediated recognition and T cell activation is indicated in Fig. 4. T cell–antigen-presenting or target cell contact is first stabilized by adhesion molecules (Fig. 4A). Because of membrane fluidity, MHC molecules with the correct peptide would congregate at the interface [as first discussed in (*23*)] (Fig. 4B). This would promote a high local density of TCR-CD3 complexes, leading to T cell activation. TCR-CD3 complexes, together with CD4 or CD8, have been observed at the interfaces of T cells and presenting cells (*23*) and T cell activation can be triggered by cross-linking TCR-CD3 molecules with antibodies (*30*). Activation results in a transient increase in adhesion (*31*) and the directional secretion of specific molecules by the T cell (*23*) (Fig. 4C). Down regulation of adhesion molecules would then permit cell detachment (Fig. 4D). Activation induces increased adhesion of T cells (*31*), which may help to explain their faster response time to antigenic challenge, and the relative resistance of naive T cells to stimulation (*32*). It also suggests that specific adhesion molecule–ligand interactions could be important in encouraging or discouraging various T cell subset–presenting cell interactions.

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Identification of a Zinc Finger Protein That Inhibits IL-2 Gene Expression

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Transient activation of the *interleukin-2* (*IL-2*) gene after antigen recognition by T lymphocytes is crucial for subsequent T cell proliferation and differentiation. Several *IL-2* gene regulatory elements and binding factors necessary for activation of the IL-2 gene have been defined. However, little is known about negative regulation of IL-2 expression, which is likely to be important in the rapid shut-off of IL-2 transcription. A nucleotide sequence element (NRE-A) that negatively regulates IL-2 expression has been identified within the IL-2 gene. T cell nuclear extracts contained an NRE-A binding activity. A complementary DNA was isolated that encodes a zinc finger–containing protein that suppressed IL-2 gene expression. The observation of negative regulation of the immunoglobulin heavy chain gene enhancer by an element similar to NRE-A suggests that related proteins may regulate multiple immune response genes.

TRANSCRIPTIONAL ACTIVATION OF the *IL-2* gene is critical for the immune response that follows stimulation of T lymphocytes via the T cell antigen receptor or the CD2, CD28, and interleukin-1 cell surface receptors (*1*). While several DNA sequence elements and binding proteins that participate in transcriptional activation of *IL-2* have been identified, little is known about how this gene is negatively regulated (*2*). Repression of *IL-2* transcription is likely to be important for explaining its transient, inducible, and tissue-specific expression.

Regulation of *IL-2* expression may be studied with the use of the human Jurkat T cell line transfected with hybrid plasmid constructs in which the *IL-2* promoter-enhancer is linked to the bacterial chloramphenicol acetyl transferase (CAT) (*3*) or firefly luciferase (Luc) (*4*) reporter genes. In transient expression assays, IL-2–reporter gene constructs [pIL2(−548)CAT and pIL2(−548) Luc] that contain IL-2 gene sequences extending 548 bp 5′ of the transcription start site display behaviors characteristic of the endogenous IL-2 gene. That is, they are inducible by T cell activation signals only in T lymphoid cells, are silent in unstimulated cells, are inhibitable by cyclosporin A (Cs-A), and employ the proper transcription start site (*3–7*).

The proximal IL-2 promoter extending 130 nucleotides 5′ of the TATA box is essential for induced IL-2 gene expression (*3, 5–7*). This region contains an inducible DNAse I hypersensitive site (*3*) near or at an Oct-1 binding site (*8*) and a binding site for Fos-Jun family heterodimers (*9*). We focused on a sequence (−110 to −101) between the Oct-1 and Fos-Jun binding sites. Using pIL2(−548)CAT and pIL2(−548)-Luc, we generated internal deletion and site-specific mutations within this region. One mutation, pIL2(−106/−101), deleted four nucleotides (residues −105 to −102), replacing them with an 8-bp Xba I linker. A second construct, pIL2(−110/−101), has eight IL-2 nucleotides deleted (residues −109 to −102) and replaced with an 8-bp linker. We transfected these constructs into Jurkat T cells to assess their transient expression in response to T lymphocyte activation signals (Fig. 1A). Mutations of the −110 to −101 region yielded three- to sevenfold increases in Luc and CAT activity in phytohemaglutinin (PHA-P)– and 12-O-tetradecanoylphorbol-13-acetate (TPA)–activated Jurkat cells, suggesting the presence of a negative regulatory element (NRE-A). The enhanced activity seen with alteration of the NRE-A region is not likely

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