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Altered T Cell Receptor Ligands Trigger a Subset of Early T Cell Signals

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Summary

TCR ligands are complexes of peptides and MHC proteins on the surfaces of APCs. Some of these ligands cause T cell proliferation (agonists), while others block it (antagonists). We compared the acid release, calcium flux, and proliferation response of helper T cells to a variety of ligands. We found that all agonist ligands but not most antagonist ligands trigger acid release, a general indicator of early cellular activation. Only a subset of ligands triggering acid release cause sustained calcium flux, and only a subset of these ligands cause T cell proliferation. Antagonist ligands and anti-CD4 antibodies both effectively block T cell proliferation. However, significantly greater antagonist ligand or antibody concentrations are required to block acid release and initial calcium influx. These data demonstrate a hierarchy of early T cell signaling steps and show that altered TCR ligands can initiate some steps while blocking the completion of others.

Introduction

Activation of helper T lymphocytes is a critical step in both humoral and cell-mediated immune response. Helper T cell activation involves ligation of multiple receptors on the T cell surface. These include two proteins, the $\alpha\beta$ T cell receptor (TCR) and the coreceptor CD4, which bind to complexes of antigenic peptides and class II major histocompatibility complex (MHC) proteins. The $\alpha\beta$ TCR binds to the region of the peptide–MHC complex surrounding the antigenic peptide, while CD4 binds to a nonpolymorphic region of MHC. Peptide–MHC complexes, as well as the ligands for many other T cell surface receptors, are membrane proteins on the surfaces of antigen-presenting cells (APCs).

Binding of peptide–MHC (henceforth referred to as ligand or TCR ligand) to the TCR triggers a series of intracellular biochemical reactions in the T cell. These reactions have been the subject of intense study (reviewed by Weiss and Littman, 1994). The first reactions, occurring within seconds, include tyrosine phosphorylation of the TCR-associated CD3 and ζ proteins. These reactions result in recruitment of the tyrosine kinase ZAP-70 to the TCR and activation of the phosphatidylinositol pathway. This pathway, which triggers increased intracellular calcium and protein kinase C (PKC) activation, is sufficient to cause, at least in some cases, interleukin-2 (IL-2) production and T cell proliferation (Kaibuchi et al., 1985; Truneh et al., 1985; Desai et al., 1990).

In addition to these reactions, ligand–TCR binding also causes colocalization of the TCR and coreceptor CD4 at the T cell–APC interface (Kupfer et al., 1987). It has recently been shown that binding of the coreceptor CD8 to class I MHC enhances the affinity of class I MHC ligand–cytotoxic TCR interaction by reducing ligand dissociation rate (Luescher et al., 1995). It is likely that CD4 similarly prolongs the duration of helper TCR ligation by binding to class II MHC. In addition, CD4 may play a role in intracellular signaling events. The cytoplasmic tail of CD4 (like the cytoplasmic tail of CD8) is associated with the tyrosine kinase $p53^{lck}$ (Lck), and this association contributes to antigen-specific signal transduction (Zamoyska et al., 1989; Chalupny et al., 1991; Glaichenhaus et al., 1991; Abraham et al., 1991; Straus and Weiss, 1992; Xu and Littman, 1993; Díez-Orejas et al., 1995). Approximately 5-fold overexpression of CD4 that lacks a cytoplasmic tail and thus fails to bind Lck is required to restore helper T cell function in CD4 knockout mice (Killeen and Littman, 1993).

Variants of antigenic TCR ligands can easily be produced by mutating the peptide portion of the ligand. Such altered ligands can produce a wide range of biological effects (reviewed by Jameson and Bevan, 1995). These include production of an altered cytokine profile (Evavold and Allen, 1991; Windhagen et al., 1995), target cell lysis without cytokine production (Evavold et al., 1995), induction of clonal anergy (Sloan-Lancaster et al., 1993), and antagonism of response to wild-type antigen (De Magistris et al., 1992). In addition, some altered ligands can block some aspects of T cell response, while triggering others (Racioppi et al., 1993; Ruppert et al., 1993). In this report, we will refer to ligands that cause T cell proliferation at low concentration as agonist ligands. Ligands that never cause T cell proliferation but can block proliferation response to agonist ligand will be referred to as antagonist ligands. Ligands with some agonist and some antagonist properties will be referred to as partial agonist ligands. We should point out that the use of these pharmacological terms to describe TCR ligands is not strictly accurate, as TCR activation is not adequately described by classical affinity models for receptor activation (Matsui et al., 1994). However, we feel that these terms nevertheless provide a useful qualitative description of T cell responses to altered ligands.

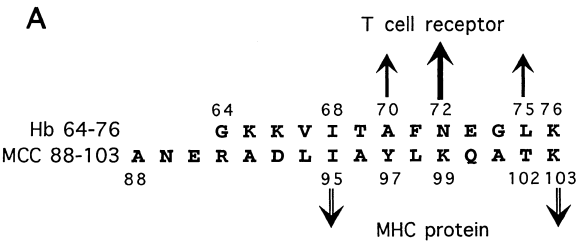
A particular difficulty in discussing TCR antagonist ligands is that peptides can block T cell activation at two different steps. First, any peptide that binds to MHC (regardless of its TCR contacts) can competitively inhibit the presentation of antigenic peptide (Ishioka et al., 1994). Second, peptides with the appropriate TCR contacts can form antagonist ligands, which block response to preformed agonist peptide–MHC complexes (De Magistris et al., 1992). In this report, we will focus exclusively on this second type of antagonism, TCR antagonism.

TCR antagonism is of particular interest for several reasons. Antagonist ligands may be involved in positive selection of T cells in the thymus (Hogquist et al., 1994). Mutant viral proteins containing peptides that form antagonist ligands may allow viral escape from immune response, resulting in persistent viral infection (Klenerman et al., 1994; Bertoletti et al., 1994). Synthetic peptides that form antagonist ligands may be used as drugs for specific therapy of autoimmune disease (Kuchroo et al., 1994; Franco et al., 1994). Moreover, TCR antagonist ligands may act via a novel biochemical mechanism.

Three pieces of evidence strongly suggest that antagonist ligands are not classical competitive antagonists. First, some antagonist ligands are active at very low concentration (10^2 per APC), while the TCR is present at high concentration (10^4 per T cell) (Harding and Unanue, 1990; De Motz et al., 1990; Christianick et al., 1991; Klenerman et al., 1994; Bertoletti et al., 1994). Second, the measured affinity of some antagonist ligands for the TCR in solution is very low (approximately one order of magnitude weaker than antigenic ligand–TCR binding) (Alam et al., 1996; Lyons et al., 1996). Thus, it would be impossible for the low concentrations of antagonist ligands that are biologically active to block receptor occupation by antigenic ligand directly. Finally, antagonist ligands themselves cause partial CD3 ζ phosphorylation (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). These observations suggest that antagonist ligands trigger a series of biochemical events that somehow render T cells less responsive to antigen.

To investigate this mechanism in greater detail, we measured the ability of altered antigenic peptides presented on live APCs to trigger T cell acid release, calcium flux, and proliferation. Calcium flux and acid release are complimentary assays that both measure T cell activation within minutes of receptor ligation (Weiss et al., 1984a, 1984b; Nag et al., 1992; McConnell et al., 1995). Acid release (as measured on a microphysiometer) is a general marker of cellular activity. Activation of most known signal transduction pathways can trigger increases in acid release (McConnell et al., 1992). Such increases may reflect either increased production of acidic metabolites (e.g., lactate via glycolysis) or activation of membrane proton transport proteins (e.g., the sodium–proton antiport) (McConnell et al., 1992; Wada et al., 1993). Intracellular calcium is a crucial early second messenger, which is directly involved in T cell activation (Kaibuchi et al., 1985; Truneh et al., 1985; Desai et al., 1990; Weiss et al., 1984a, 1984b). In contrast with acid release, calcium flux can be recorded in single cells as opposed to a bulk population of cells (Donnadieu et al., 1992, 1994; Rötnes and Bogen, 1994; Torigoe et al., 1995; Agrawal and Linderman, 1995).

In this report, we compare T cell acid release, calcium flux, and proliferation in response to different combinations of agonist and antagonist ligands. We describe a hierarchy of T cell responses in which acid release and initial calcium flux are the easiest to trigger and proliferation is the most difficult. The existence of such a hierarchy indicates that early T cell signaling occurs in multiple (although not necessarily sequential) steps. The effect of antagonist ligands and anti-CD4 antibodies is to shift



B

Hb	GKKVITAFNEGLK	agonist
74A	----------A--	partial agonist
70M	------M------	antagonist
70S	------S------	antagonist
73D	---------D---	antagonist
72Q	--------Q----	inactive
MCC	ANERADLIAYLKQATK	agonist
102S	--------------S-	partial agonist
102G	--------------G-	antagonist
99R	----------R----	antagonist

Figure 1. Hb and MCC Peptide Sequences

(A) Probable alignment of these peptides when presented on the class II MHC protein I-E^k. The recent x-ray crytstal structure of the Hb peptide bound to I-E^k confirms that position 68 of Hb (isoleucine) occupies the P1 pocket with position 76 (lysine) in the P9 pocket (Fremont et al., 1996).

(B) Name, sequence, and biological function of the altered peptides studied in this report.

agonist ligands to a lower level on the signaling hierarchy. We conclude by discussing the implications of these findings for models of TCR antagonism.

Results

PL.17 T Cell Acid Release Response to Altered Hemoglobin Ligands

The PL.17 T cell clone is triggered by the peptide corresponding to residues 64–76 of the hemoglobin–β^d protein (the Hb peptide) bound to the MHC protein I-E^k. The alignment of the Hb peptide in the MHC-binding grove of I-E^k, and the physiologic and biochemical responses of the T cell clone PL.17 to altered Hb ligands, have been extensively evaluated in prior work (as summarized in Figure 1; Evavold et al., 1992, 1994, 1995; Sloan-Lancaster et al., 1993, 1994; Fremont et al., 1996). Therefore, this system was ideal for comparing acid release to traditional measures of T cell activation.

We measured the acid release response of a mixture of PL.17 T cells and APCs to previously studied single amino acid mutants of the Hb peptide (Figure 1B). Increased acid release is triggered by both Hb and partial agonist 74A at low peptide concentration (Figure 2A). None of the antagonist ligands trigger acid release at low concentration, but one (70M) markedly increased acid release at high concentration (Figure 2). This antagonist has also been shown to cause transient spikes in intracellular calcium in single cell calcium flux assays (J. Sloan-Lancaster and P. M. A., unpublished data). Inactive ligand 72Q does not trigger acid release. Although only one of the Hb antagonist ligands triggers significant acid release at the peptide concentrations studied in this report, 70M and 70S both cause partial

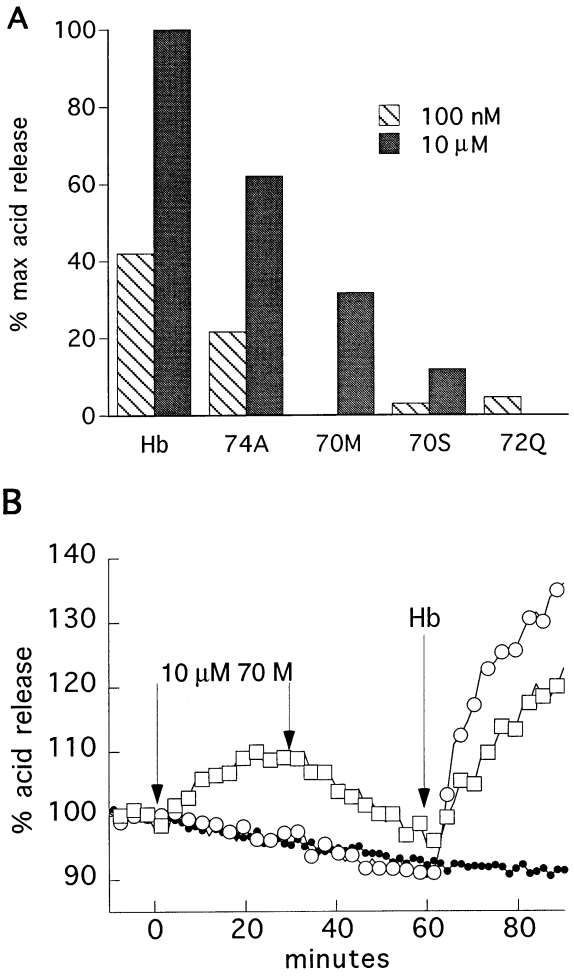


Figure 2. PL.17 Acid Release Response to Altered Hb Ligands

(A) Acid release response to agonist and antagonist ligands. The rate of acid release of a mixture of PL.17 T cells and APCs was measured before and after a 30 min exposure to the indicated peptides. The increase in acid release triggered by these ligands is reported as a percentage of the increase triggered by 10 μM wild-type Hb (30%). APCs were CHO cells transfected with I-E^k. A similar response was obtained using CH27 cells as APCs in an independent experiment.

(B) Antagonist ligand 70M can both trigger and block PL.17 T cell acid release. The rate of acid release of a mixture of PL.17 T cells and CH27 APCs was measured every 90 s. Hb antagonist 70M (10 μM) was added during the interval indicated by the two shorter arrows, and unbound peptide was washed away. Wild-type Hb (1 μM) was added at the time indicated by the long arrow for the remainder of the experiment. Squares received 70M peptide and Hb; circles did not receive 70M (open circles received Hb; closed circles are negative control). Effect of another antagonist (73D) was also measured in this experiment (data not shown). 73D alone resulted in only a very slight increase in acid release, but 73D was as effective as 70M in blocking response to Hb.

CD3 ζ chain phosphorylation and can induce long-lasting T cell anergy (Sloan-Lancaster et al., 1994). Therefore, all antagonist ligands may trigger some early T cell signaling, but this signaling does not always cause acid release.

To investigate the mechanism by which antagonist ligands block T cell activation, we measured the ability of altered Hb ligands to inhibit acid release triggered by Hb. We first measured response to 1 μM Hb, added either simultaneous with, or up to 2 hr following, addition of 10 μM 70S or 72Q. Antagonist ligand 70S, but not inactive ligand 72Q, caused a 20%–60% reduction in PL.17 acid release if added either simultaneous with or prior to Hb (three independent experiments, data not shown). These results raised the intriguing possibility that the antagonist ligand 70M might be able both to trigger, and to block, PL.17 acid release. Indeed, while 70M alone causes some acid release, addition of 70M followed by Hb results in reduced T cell acid release compared with addition of Hb alone (Figure 2B). The ability of antagonist ligands to block acid release is consistent with previous results demonstrating that antagonist ligands block other early T cell signals such as T cell phosphatidylinositol hydrolysis and calcium flux (Ruppert et al., 1993).

5C.C7 T Cell Response to Altered Moth Cytochrome C Ligands Reveals More Stringent Requirements for Proliferation than for Acid Release or Calcium Flux

The 5C.C7 T cell line is derived from $CD4^+$ spleen cells from a 5C.C7 TCR transgenic mouse (Fink et al., 1986; Seder et al., 1992). It is triggered by the peptide corresponding to residues 88–103 of the moth cytochrome c protein (the MCC peptide) bound to the MHC protein I-E^k. The effect of mutation of the MCC peptide on IL-3 production by a partially transformed 5C.C7 T cell clone has been extensively evaluated (Reay et al., 1994). In addition, the dissociation rate of altered MCC ligands from the 2B4 TCR (which is similar in sequence and ligand specificity to the 5C.C7 TCR) has been measured in solution and correlates with T cell biological response (Matsui et al., 1994; Lyons et al., 1996). Based on these studies, we chose to investigate 5C.C7 T cell acid release and calcium response to three single amino acid mutants of MCC with previously demonstrated biological activities (see Figure 1B; Reay et al., 1994; Lyons et al., 1996; P. A. Reay and M. M. D., unpublished data). A variety of other peptides (99Q, 99E, 99A, 97I, 97Q, and triple mutant 93E 99T 102A), which were neither effective agonists nor antagonists of 5C.C7 IL-3 production in previous studies (Reay et al., 1994; P. A. Reay, unpublished data), were included for comparison. The mutations studied are in TCR contact residues (see Figure 1A). All of these peptides form stable complexes with I-E^k (Reay et al., 1994).

Only one of these altered ligands, 102S, a conservative single amino acid mutation (T→S) at a minor T cell contact, results in 5C.C7 T cell proliferation (Figure 3A). Moreover, 102S is the only altered MCC ligand to trigger marked acid release (Figure 3B). While the proliferation dose response to partial agonist 102S and wild-type MCC is very different, the acid release dose response is almost identical (Figure 3). Moreover, no differences in acid release kinetics were noted between these two ligands (Figure 3C; for a detailed analysis of MCC response kinetics see Beeson et al., 1996). Thus, it appears that the requirements for triggering acid release

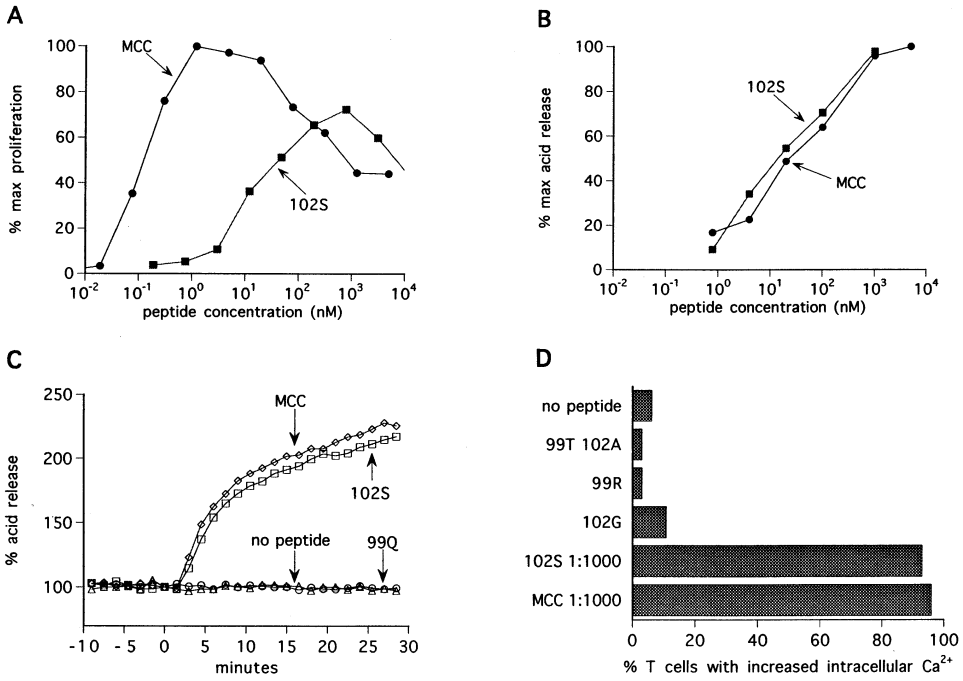


Figure 3. Comparison of 5C.C7 Proliferation, Acid Release, and Calcium Flux Response to Altered MCC Ligands

(A) MCC, but not partial agonist 102S, triggers proliferation at low concentration. Responses are reported as a percentage of maximum [^{3}H]thymidine incorporation in response to MCC (160×10^3 cpm). B10.BR mouse splenocytes were used as APCs. No increase in [^{3}H]thymidine incorporation above background (10^3 cpm) was found in response to MCC mutants 102G, 99R, 99E, 97I, or 97Q at concentrations up to 50 μM. Data are representative of five independent experiments.

(B) Both MCC and 102S trigger acid release at low concentration. The rate of acid release of a mixture of 5C.C7 T cells and APCs was measured before and after 30 min exposure to the indicated peptides. 102S and MCC dose response curves were measured in separate experiments, using response to 5 μM MCC (92% and 128%, respectively) as a standard for comparison. APCs were CHO cells transfected with I-E^k. Similar acid release response to MCC and 102S was observed in greater than five independent experiments. Acid release response to 102G, 99R, 99E, 97I, and 97Q was <10% of response to 1 μM MCC in all experiments using these peptides (all peptides were tested in at least two experiments at concentrations up to 10 μM).

(C) Kinetics of acid release response to MCC and 102S are similar. At t = 0 min, 1 μM of the indicated peptides was added to a mixture of 5C.C7 T cells and CH27 cells continuously for the duration of the experiment. Plotted is the rate of acid release as a percentage of the acid release prior to the addition of peptide.

(D) Both MCC and 102S trigger increases in intracellular calcium at low concentration. Plotted is the percentage of 5C.C7 cells demonstrating at least transient increases in intracellular calcium in response to APCs preincubated with MCC or altered MCC peptides. MCC and 102S were diluted 1:1,000 into the inactive peptide 93E 99T 102A. 102G and 99R were not diluted. APCs were CHO cells transfected with I-E^k.

are more easily fulfilled than those for triggering proliferation. This is consistent with the ability of the Hb antagonist ligand M70 to trigger acid release but not proliferation.

We also measured the single cell calcium flux response of 5C.C7 T cells to APCs preloaded with various combinations of altered MCC ligands. In all experiments, the biologically active peptides of interest were diluted into the inactive peptide 93E 99T 102A to yield a total peptide concentration of 25 μM for APC loading. APCs were washed to remove all free peptide prior to the addition of T cells. A 1:1,000 dilution of MCC or 102S (an MCC or 102S concentration of 25 nM) results in increased intracellular calcium in >90% of 5C.C7 T cells (Figure 3D). However, MCC and 102S trigger qualitatively different patterns of calcium flux, with 102S resulting in some transient calcium flux responses (data not shown). These transient responses are characterized by spikes to high intracellular calcium, followed by a return to basal levels. Some cells demonstrating transient responses returned to basal levels within seconds, while others remained at elevated calcium concentrations for minutes. Undiluted 102S resulted in a similar pattern of calcium response to 102S diluted 1:1,000 (data not shown), demonstrating that calcium flux, in contrast with acid release, depends only weakly on peptide dose. Comparing the acid release, calcium flux, and proliferative responses to 102S reveals that transient calcium flux correlates with full acid release (Figure 3B) but only minimal proliferation (Figure 3A). In contrast with the strong calcium response to agonist or partial agonist at 1:1,000 dilution, even the undiluted antagonists 102G or 99R give no calcium flux in most cells. However, a transient calcium flux was observed in >10% of 5C.C7 cells in response to 102G (Figure 3D).

Altered MCC Ligands Are More Efficient Antagonists of 5C.C7 Proliferation than of Acid Release or Calcium Flux

To determine which of the altered MCC ligands are TCR antagonists, we measured the ability of 102S, 102G, 99R, and 99E to block 5C.C7 proliferation triggered by

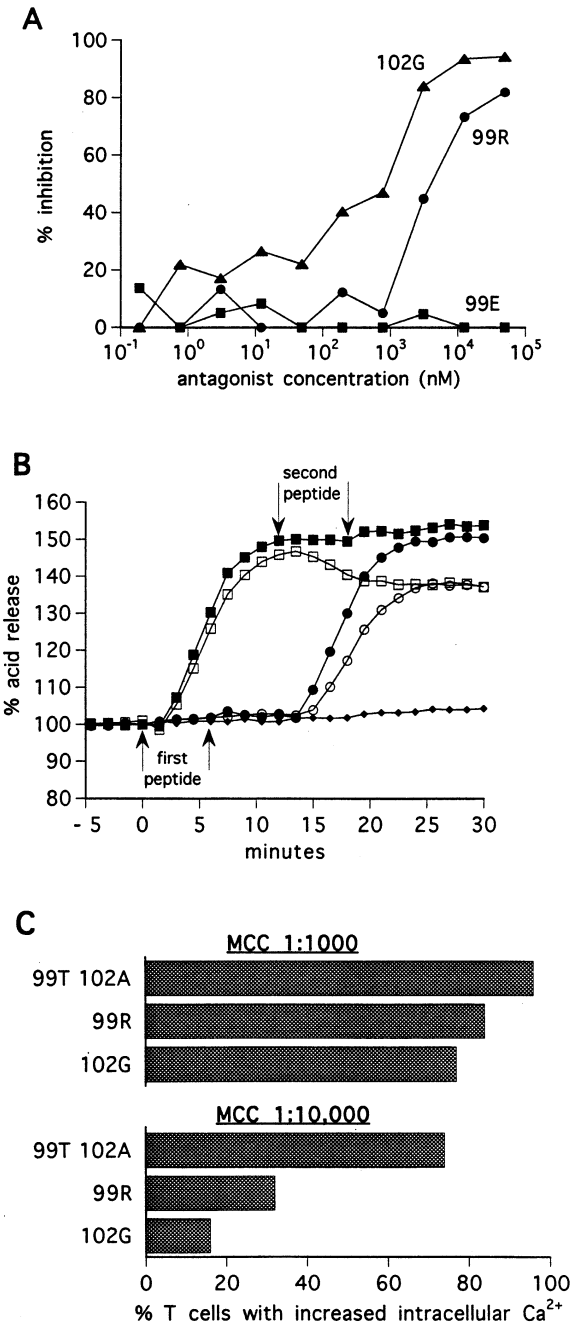


Figure 4. Antagonism of 5C.C7 T Cell Proliferation, Acid Release, and Calcium Flux by Altered MCC Ligands

(A) 102G and 99R are effective antagonists of 5C.C7 proliferation. 5C.C7 T cells were incubated with the indicated peptides and APCs prepulsed for 2 hr with 200 nM MCC. Proliferation was measured based on [^{3}H]thymidine incorportation. Prepulsed APCs alone resulted in [^{3}H]thymidine incorporation of 31 $\times$ 10^3 cpm. B10.BR mouse splenocytes were used as APCs. Data are representative of three independent experiments.

(B) 99R slightly inhibits 5C.C7 acid release when added either before or after addition of MCC. Mixtures of 5C.C7 T cells and CHO cells transfected with I-E^k were treated with two different peptides sequentially as indicated by the arrows: 50 nM MCC followed by 10 μM 99E (closed squares), 50 nM MCC followed by 10 μM 99R (open squares), 10 μM 99E followed by 50 nM MCC (closed circles), 10 μM 99R followed by 50 nM MCC (open circles). Small diamonds are the negative control and received no peptide. Plotted is the rate of acid release as a percentage of the rate prior to addition of peptide.

(C) A vast excess of 99R or 102G is required to block fully calcium flux response to MCC. 5C.C7 T cells were exposed to APCs preincubated with wild-type MCC diluted either 1:1,000 or 1:10,000 into inactive peptide 93E 99T 102A or antagonist peptide 99R or 102G. Plotted is the percentage of T cells showing increases in intracellular calcium. APCs were CHO cells transfected with I-E^k.

a suboptimal concentration of MCC in a standard prepulse antagonism assay (De Magistris et al., 1992). Not surprisingly, increasing the concentration of 102S enhances proliferation (data not shown). In contrast, 102G and 99R (but not 99E) are effective antagonists of T cell proliferation when added in 10- to 50-fold excess of MCC (Figure 4A).

To investigate how 99R and 102G block 5C.C7 proliferation, we measured their ability to block 5C.C7 calcium flux and acid release. As shown in circles in Figure 4B, addition of a 200-fold excess of 99R but not 99E prior to addition of a suboptimal amount of MCC inhibits 5C.C7 acid release. This parallels inhibition of PL.17 response to Hb by addition of antagonist prior to agonist. To determine whether the sequence of addition of agonist and antagonist is important, we also added 99R and 99E to cells that had already been triggered by MCC. As shown in squares in Figure 4B, addition of an antagonist peptide rapidly decreases 5C.C7 acid release. Thus, acid release depends on the relative amounts of agonist and antagonist ligands present at any given time, regardless of the order of addition. Another interesting aspect to this experiment is the relatively small decrease caused by the 200-fold excess of antagonist (10 μM). This excess of antagonist is sufficient to block most 5C.C7 cell proliferation.

The effect of antagonists on calcium flux is consistent with their effects on proliferation and acid release. As shown in Figure 4C, the presence of even a 1,000-fold excess of 102G or 99R relative to MCC has only a slight effect on the percentage of cells giving a positive calcium flux response. However, antagonist ligands are capable of converting agonist-type calcium flux (full signals in all cells) to partial agonist type calcium flux (transient flux in some cells) (data not shown). To study further the effect of antagonist on calcium flux, we reduced the agonist ligand concentration 10-fold, while keeping the antagonist ligand concentration the same. In this case, both 102G and 99R greatly reduce the frequency of a positive calcium flux responses (Figure 4C). However, even this large excess of antagonist (10,000-fold) does not eliminate all calcium flux. Thus, it appears that blocking calcium flux, like blocking acid release, is more difficult than blocking proliferation. This result is in contrast with previous work demonstrating that the ability to block calcium flux and proliferation are strongly correlated (Ruppert et al., 1993). A likely explanation for this discrepancy is that Ruppert et al. measure bulk, rather than single cell, calcium flux.

An Anti-CD4 Antibody Converts a Partial Agonist into an Antagonist, Despite Having a Minimal Effect on Acid Release and Calcium Flux

Anti-CD4 antibodies can block helper T cell activation both in vitro and in vivo (Dialynas et al., 1983; Waldor

et al., 1985). We were interested in examining the effect of the anti-CD4 antibody GK1.5 on T cell response to different altered ligands. We first measured the ability of anti-CD4 to block T cell proliferation. Figure 5 shows the effect of varying concentrations of anti-CD4 on 5C.C7 proliferation. Plotted is dose response to wild-type MCC (Figure 5A) or partial agonist 102S (Figure 5B) in the presence of a fixed concentration of anti-CD4. A low concentration of anti-CD4 (100 ng/mL) only slightly inhibits proliferation in response to MCC but completely eliminates the response to 102S. A 10-fold greater concentration of anti-CD4 blocks most but not all proliferation caused by MCC. GK1.5 Fab fragments produce similar results (data not shown), demonstrating that the effect of GK1.5 does not require cross-linking of CD4.

The ability of wild-type MCC but not partial agonist 102S to trigger proliferation in the presence of 100 ng/mL anti-CD4 allows the measurement of 102S antagonist function. As shown in Figure 5C, in the presence of anti-CD4 and MCC, increasing concentrations of 102S block 5C.C7 proliferation. Because the APCs are prepulsed with MCC, this blockade cannot be due to competition for MHC binding and must result from a TCR specific effect. Thus, anti-CD4 converts a partial agonist into a TCR antagonist. A similar result has been previously reported for $CD8^+$ T cells (Jameson et al., 1994).

To investigate the mechanism by which anti-CD4 blocks proliferation, we measured 5C.C7 acid release and calcium flux in the presence of anti-CD4. In control experiments, we found that in the absence of peptide, anti-CD4 does not trigger acid release or calcium flux (data not shown). Figure 6A shows that the acid release response to 102S is not inhibited by a concentration of anti-CD4 (100 ng/mL), which completely blocks proliferation response. Figure 6B summarizes the effect of anti-CD4 on calcium flux response to MCC and 102S. While 1 μg/mL anti-CD4 does not completely block calcium flux response to MCC or 102S, it tends to convert calcium flux from an agonist to a partial agonist pattern (data not shown).

Discussion

Defining a Hierarchy of Early T Cell Responses

Binding of the TCR and CD4 coreceptor protein to their ligand, a complex of an antigenic peptide and a class II MHC protein, activates helper T cells. Binding of an altered ligand may cause partial T cell activation, block T cell response to agonist ligand, or both. To investigate the molecular mechanism of this differential T cell response, we measured the pattern of early T cell signaling by agonist, partial agonist, and antagonist ligands.

T cell acid release was triggered by all agonist and partial agonist ligands but by only one of the five antagonist ligands studied in this report (as summarized in Figure 7). Because acid release is triggered by most known signal transduction pathways (McConnell et al., 1992), this suggests that many antagonist ligands do not trigger the production of large amounts of second messengers. However, one ligand that induces T cell anergy did not trigger detectable acid release, demonstrating that acid release cannot detect all T cell signaling. Partial ζ phosphorylation is one event triggered by

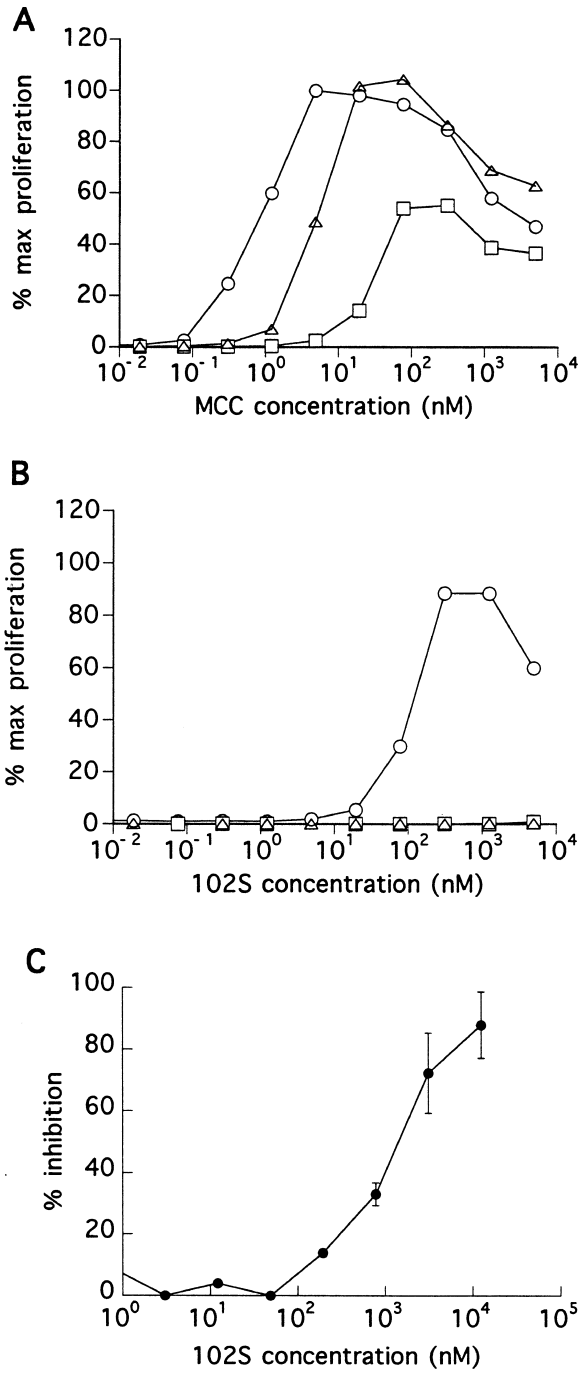


Figure 5. Anti-CD4 Antibody GK1.5 Converts Partial Agonist 102S into a T Cell Receptor Antagonist

(A) 5C.C7 proliferation dose response to MCC in the presence of 1 μg/mL (squares), 100 ng/mL (triangles), or no (circles) anti-CD4 antibody GK1.5. Responses are reported as a percentage of maximum [^{3}H]thymidine incorporation in absence of anti-CD4 (164 × 10^3 cpm).

(B) Identical experiment to (A), using 102S in place of MCC.

(C) Antagonism of 5C.C7 T cell proliferation by 102S. 5C.C7 T cells were incubated with the indicated concentration of 102S, 100 ng/mL anti-CD4 antibody GK1.5, and APCs prepulsed for 2 hr with 200 nM MCC. Prepulsed APCs alone resulted in [^{3}H]thymidine incorporation of 105 × 10^3 cpm. Assays were performed in triplicate. Plotted is mean inhibition of [^{3}H]thymidine incorporation ± standard error. B10.BR splenocytes were used as APCs in (A), (B), and (C).

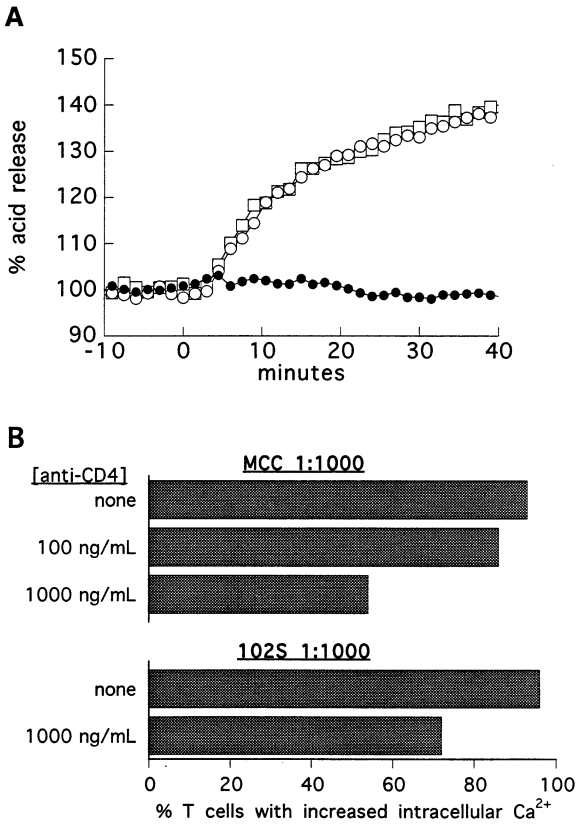


Figure 6. Anti-CD4 Antibody GK1.5 Only Slightly Alters 5C.C7 Acid Release and Calcium Flux Responses

(A) GK1.5 does not affect either the magnitude or the kinetics of acid release response to 1 μM 102S. Between t = 0 min and t = 30 min, either 1 μM 102S plus 100 ng/mL GK1.5 (squares) or 1 μM 102S alone (open circles) was added to a mixture of 5C.C7 T cells and CH27 APCs. Closed circles are negative control (no peptide added). Plotted is the rate of acid release as a percentage of the rate prior to addition of peptide.

(B) Effect of GK1.5 on 5C.C7 calcium flux response. 5C.C7 T cells were exposed to APCs preincubated with either MCC or 102S (diluted 1:1,000 into inactive peptide 93E 99T 102A). The percentage of T cells responding with increases in intracellular calcium was measured in the presence of the indicated concentrations of anti-CD4 antibody GK1.5. APCs were CHO cells transfected with I-E^k.

antagonist ligands that probably does not significantly increase acid release.

We found that one partial agonist ligand (102S), which is inefficient at causing proliferation, is nevertheless very effective at triggering acid release (Figure 3). This dichotomy demonstrates that there is not a one-to-one correspondence between a given level of acid release and later T cell responses. It is reminiscent of the ability of a different altered ligand to cause IL-4 but not IL-2 production (Evavold and Allen, 1991). We found that ligands like 102S, which trigger acid release but not proliferation, result in increased intracellular calcium in most T cells. However, such ligands result in transient, rather than sustained, increases in intracellular calcium in many cases. Transient calcium fluxes triggered by suboptimal TCR activation have been previously shown to result in deficient IL-2 production (Goldsmith and Weiss, 1988).

A

stimulus	phospho-ζ	H+	prolif.
Hb	++	++	++
74A	N.D.	++	+
70M	+	+	-
73D	N.D.	+/-	-
70S	+	-	-
72Q	-	-	-

B

stimulus	partial calcium	H+	full calcium	prolif.
MCC	++	++	++	++
MCC + anti-CD4	++	++	+	+
102S	++	++	+	+
102S + anti-CD4	++	++	+	-
MCC + 102S + anti-CD4	++	N.D.	+	-
MCC + 102G	+	+	-	-
MCC + 99R	+	+	-	-
102G	+/-	-	-	-
99R	-	-	-	-

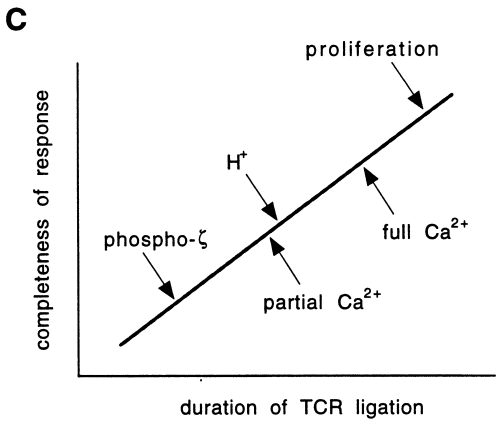


Figure 7. Defining a Hierarchy of Steps in T Cell Activation

(A) Summary of PL.17 T cell responses to altered Hb ligands. The number of plus signs refers to the strength of a particular response. Minus signs indicate no response. ζ chain phosphorylation data are taken from Evavold et al. (1994). In the case of ζ phosphorylation, a single plus indicates predominant production of the p21 isoform, while two plus signs indicate significant production of the p23 isoform.

(B) Summary of 5C.C7 T cell responses to various combinations of altered MCC ligands.

(C) Schematic depicting proposed relationship between the completeness of a T cell response and the duration of TCR ligation by peptide–MHC complex. Arrows depict where certain T cell responses might be located on such a plot, based on data in (A) and (B). Phospho-ζ arrow placement is based on the ease of p21 ζ chain phosphorylation. Phospho-ζ, ζ chain phosphorylation; H$^+$, acid release; Ca^{2+}, calcium flux.

As a unit, and in combination with previous biochemical studies (Sloan-Lancaster et al., 1994; Madrenas et al., 1995), these results suggest a hierarchy of measures of T cell activation (Figure 7). At the bottom of the hierarchy is initial CD3 ζ chain phosphorylation, as it is triggered by ligands that failed to trigger any detectable response in this report (e.g., 70S, Figure 2). Just above

ζ phosphorylation are initial calcium flux and acid release, as these responses are triggered by a large number of different stimuli, many of which fail to yield T cell proliferation. Of these two assays, calcium flux is slightly more sensitive (e.g., 102G alone results in a very small but detectable calcium flux response but no detectable increase in acid release). This is probably because a single cell assay (calcium flux) is more effective than an assay of bulk cellular response (acid release) for detecting a small response in a small fraction of cells. Higher up are various types of sustained increases in intracellular calcium, which can be subdivided based on the precise temporal pattern of T cell response (C. W. et al., unpublished data). At the top of the hierarchy is T cell proliferation, which requires nearly ideal activation conditions. These conditions include the appropriate APC and microenvironment (reviewed by Janeway and Bottomly, 1994), as well as the appropriate ligand. In contrast with proliferation, and in accordance with their lower location on the hierarchy, acid release and calcium flux are relatively insensitive to APC type (Beeson et al., 1996; Jenkins et al., 1987).

An important question raised by this hierarchy is whether the different responses are causally related (e.g., ζ phosphorylation causes calcium flux, which causes acid release and proliferation). Acid release but not calcium flux depends strongly on peptide dose. Moreover, acid release increases steadily for at least the first 30 min of T cell stimulation, while intracellular calcium frequently peaks rapidly and then declines within minutes of T cell activation. These disparities suggest that acid release and calcium flux, despite occuring during a similar time frame and in response to similar stimuli, are independent responses triggered by different signaling pathways. Thus, at least some of the responses on the signaling hierarchy appear not to be causally related and rather to be complementary measures of TCR signaling.

Antagonist Ligands and Anti-CD4 Antibodies Shift Agonist Ligands to a Lower Level on the Signaling Hierarchy

The ability of antagonist ligands to block early T cell signaling (Ruppert et al., 1993), but not always all cytokine production (Racioppi et al., 1993) raises the following dilemma: if early signaling is blocked, how can some cytokine production persist? The analysis of early T cell signaling in this report (as summarized in Figure 7) provides a simple solution to this dilemma. We find that antagonist ligands have a dual affect on T cell signaling when measured at the level of single cell calcium flux. This effect is to reduce both the fraction of cells responding to agonist, and the fraction of these responses that are complete. Because a mixture of complete and incomplete signals is probably sufficient for production of some but not all cytokines (Evavold and Allen, 1991; Racioppi et al., 1993; Windhagen et al., 1995), these results explain both the strong correlation between blockade of early signaling and blockade of proliferation, and the ability to block selectively the production of a subset of cytokines. As anti-CD4 antibodies have a similar effect to antagonist ligands on early T cell signaling, it is not surprising that they tend to induce a shift from a Th1 to a Th2 type cytokine profile (Stumbles and Mason, 1995).

The ability of both antagonist ligands and anti-CD4 to reduce the fraction of complete T cell signals suggests that both act to shift T cell response to a lower position on the hierarchy of signaling steps (Figure 7). Based on this idea, we predict that it will be much easier to block proliferation response to partial agonist than to wild-type agonist, as partial agonist starts at a lower position on the signaling hierarchy. Indeed, we have found that an equimolar amount of 102G is sufficient to block proliferation response to 102S (C. B., unpublished data). Thus, one possible explanation for the ability of some antagonists to block T cell response when the agonist is in molar excess (Bertoletti et al., 1994; Klenerman et al., 1994) is that the agonists used in these reports might be suboptimal.

Continuous Spectrum of T Cell Responses Supports a Kinetic Discrimination Model of T Cell Activation and Antagonism

Two main hypotheses have been proposed to account for the difference in T cell response to agonist versus antagonist ligands. These competing models can be labeled as a conformation model and a kinetic discrimination model. The conformation model claims that antagonist ligand binding results in a conformation of the TCR, which sends an incomplete or negative signal that can block response to antigen. Included in this idea are models where the shape of the antagonist ligand results in failure to recruit CD4 (Yoon et al., 1994) or failure to achieve appropriate TCR dimerization (Sette et al., 1994). In its simplest form, this model predicts a dichotomy between ligands that induce the correct conformation (agonists) and ligands that induce the other conformation (antagonists). In the kinetic discrimination model, a variant of kinetic proofreading (McKeithan, 1995), both agonist and antagonist ligands bind the TCR in an identical manner. The only difference is that agonist ligands bind to the TCR for a longer duration than antagonist ligands. In such a model, brief TCR occupation by an antagonist ligand triggers incomplete signaling that can block response to agonist (Rabinowitz et al., 1996). This model is supported by direct measurements of the dissociation rate of mutant MCC ligands for the 2B4 TCR (Matsui et al., 1994; Lyons et al., 1996) and by evidence that ligands causing partial T cell activation tend to have lower TCR affinities and faster dissociation rates than ligands triggering full T cell activation (Al-Ramadi et al., 1995; Alam et al., 1996; Margulies, 1996). It predicts that the continuous range of dissociation rates of ligands from the TCR will result in a continuous spectrum of T cell responses. The data in this report (as summarized in Figure 7) provide evidence for a continuous spectrum of T cell responses, suggesting that the kinetic discrimination model is a likely possibility.

The coreceptor CD4 contributes both to the stability of ligand–TCR interaction and (via Lck) to the intracellular events required for complete T cell signaling. Therefore, the kinetic discrimination model (as well as some conformation models in which CD4 recruitment is crucial) predicts that reduction in CD4 expression will convert some

partial agonist ligands causing T cell proliferation into ligands blocking proliferation. We tested this hypothesis using anti-CD4 monoclonal antibodies to approximate a reduction in CD4 expression. As predicted, anti-CD4 converted a partial agonist ligand into an antagonist ligand. Recent results have also shown that increasing CD4 expression can convert an antagonist into an agonist, and, most interestingly, an inactive ligand into an antagonist (Vidal et al., 1996). This final result is consistent with a kinetic discrimination model but not conformation models in which CD4 recruitment is crucial.

An unresolved question raised by the kinetic discrimination model is how a T cell integrates a mixture of complete and incomplete receptor triggering to yield a functional response. While the data in this report do not offer a full answer to this question, they demonstrate that the T cell at least partially intergrates these different receptor events early in the signaling pathway. The ability of the T cell to discriminate accurately between a large number of low affinity ligands (e.g., 99R or 102G) and a small number of moderate affinity ligands (e.g., 102S) at the stage of acid release or initial calcium flux suggests that incomplete receptor activation can block one of the earliest steps in generation of a complete receptor signal. The ability of antagonist ligand to rapidly decrease the acid release of a T cell already activated by agonist (Figure 4B) further supports this notion. These results are most easily explained by a model in which all TCR ligands trigger both positive and negative signals, with different T cell responses requiring different ratios of the two. Identification of the molecular events involved in blockade of a positive T cell response by incomplete receptor activation presents a challenge for future investigation of TCR antagonism.

Experimental Procedures

Peptides and Antibodies

Peptides were synthesized with standard FMOC chemistry, purified by reverse phase high pressure liquid chromatography, and characterized either by mass spectroscopy or amino acid content. Anti-CD4 antibody GK1.5 (anti-L3T4) was purchased from Becton-Dickinson (Bedford, Massachusetts).

Cell Lines

The MCC-specific 5C.C7 T cells were derived from the 5C.C7 $\alpha\beta$ TCR transgenic mouse (Fink et al., 1986; Seder et al., 1992). Spleen cells were primed with 10 μM MCC and expanded for 8 days in IL-2 containing medium. The T cells (1×10^6 cells/mL) were restimulated with irradiated (3,000 rads) B10.BR spleen cells and 10 μM MCC. After 10 days of culture and expansion in the presence of IL-2, $CD4^+$ cells were sorted by flow cytometry and restimulated in 10 day intervals. The Hb-specific PL.17 T cell clone was grown as previously described (Lorenz and Allen, 1989; Evavold et al., 1992, 1994). CH27 cells are an H-2^k B cell lymphoma that expresses the I-E^k/I-A^k MHC class II molecules, ICAM-1, and B7 costimulatory molecules. Chinese hampster ovary (CHO) cells transfected with I-E^k were grown as previously described (Wettstein et al., 1991).

Microphysiometry

Acid release was measured as described previously (McConnell et al., 1995). In brief, T cells rested from stimulation (9–13 days) were combined with APCs that had been cultured overnight in RPMI 1640 medium containing 2.5% fetal calf serum. Cells were mixed at a ratio of twenty T cells to one APC and collected by centrifugation. The cell pellet (3–6 $\times 10^6$ cells) was resuspended in 105 μL medium, which was mixed with 35 μL of melted low temperature–melting agarose (Molecular Devices, Sunnyvale, California) at 37°C. The agarose cell mixture (10 μL) was immediately spotted onto the membrane of a Cytosensor cell capsule (Molecular Devices, Sunnyvale, California). After 10 min, the cell capsule was assembled and loaded in the microphysiometer chamber maintained at 37°C. The chamber was perfused (50 μL/min) with low buffer RPMI 1640 medium (Molecular Devices, Sunnyvale, California) containing 1 mM sodium phosphate, 1 mg/mL endotoxin-free bovine serum albumin (Calbiochem, San Diego, California), and no bicarbonate (pH 7.4). The rate of acid release was determined with 20 s potentiometric rate measurements after a 58 s pump cycle and 10 s delay (total cycle time, 90 s). Baseline acid release rates were between 60–100 $\mu V\ s^{-1}$.

Calcium Imaging

For calcium imaging 5CC7 T cells were loaded for 30 min at room temperature with 2 μM Fura-2. CHO cells transfected with I-E^k were grown in 8-well coverslips (Nunc, Naperville, Illinois) and peptide loaded with 25 μM peptide between 4–16 hr. After washing unbound peptide away, Fura-2–loaded T cells were added, on the microscope stage, onto a patch of confluent CHO cells to start the experiment. Intracellular calcium in T cells was determined using C Imaging-1280 System hardware and the Simca Quantitative Fluorescence Analysis Software Package (both from Compix, Incorporated, Imaging Systems, Mars, Pennsylvania). The imaging system was coupled to a Nikon Diaphot 300 inverted microscope, which was equipped with the epifluorescence attachment and a 75W Xenon arc lamp. Alternate excitation of Fura-2–loaded T cells at 340 and 380 nm was achieved using a Ludl high speed dual filter wheel (Ludl Electronic Products, Hawthorne, New York) controlled by the Simca software. Images were collected with a CCD camera (Dage-MTI CCD72) in combination with a SuperGenII intensifier (Dage-MTI, Michigan City, Indiana) to amplify fluorescence. Analysis of intracellular calcium was achieved with the Simca software package. Prior to T cell activation, emission intensity at 380 nm is greater than emission intensity at 340 nm. A positive T cell calcium flux response was defined as an increase in intracellular calcium sufficient to produce a decrease in emission at 380 nm to less than emission at 340 nm for at least 20 s.

Cell Proliferation

Proliferation of 5C.C7 T cells was measured as the uptake of [^{3}H]thymidine. Rested T cells (3×10^4) and irradiated (3,000 rads) B10.BR spleen cells (5×10^5) were incubated with serial dilutions of peptides in a 96-well plate. We added 1 μCi [^{3}H]thymidine at 48 hr and cell DNA was harvested at 64 hr. Background radioactivity was <1,000 cpm.

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