Clone-specific T Cell Receptor Antagonists of Major Histocompatibility Complex Class I–restricted Cytotoxic T Cells

By Stephen C. Jameson,* Francis R. Carbone,‡ and Michael J. Bevan*

*From the *Howard Hughes Medical Institute, Department of Immunology, University of Washington, Seattle, Washington 98195; and the ‡Department of Pathology and Immunology, Monash Medical School, Prahran, Victoria 3181, Australia*

Summary

A previous report showed that the proliferative response of helper T cells to class II major histocompatibility complex (MHC)–restricted antigens can be inhibited by analogues of the antigen, which act as T cell receptor (TCR) antagonists. Here we define and analyze peptide variants that antagonize various functions of class I MHC–restricted cytotoxic T lymphocyte (CTL) clones. Of 64 variants at individual TCR contact sites of the K^b-restricted octamer peptide ovalbumin$_{257-264}$ (OVAp), a very high proportion (40%) antagonized lysis by three OVAp-specific CTL clones. This effect was highly clone specific, since many antagonists for one T cell clone have differential effects on another. We show that this inhibition of CTL function is not a result of T cell–T cell interaction, precluding veto-like phenomena as a mechanism for antagonism. Moreover, we present evidence for direct interaction between the TCR and antagonist–MHC complexes. In further analysis of the T cell response, we found that serine esterase release and cytokine production are susceptible to TCR antagonism similarly to lysis. Ca^{2+} flux, an early event in signaling, is also inhibited by antagonists but may be more resistant to the antagonist effect than downstream responses.

For mature T cells, stimulation of the TCR by an appropriate peptide–MHC complex on an APC usually results in activation. However, recent data have demonstrated that certain variants of the antigenic peptide can inhibit the proliferative response of helper T cells to antigen (1). Those experiments showed that this effect was not due to blockade of the restricting MHC molecule by the variant peptide, a form of peptide inhibition that has been well described (2–4), and indicated that the peptide variants acted as TCR antagonists, i.e., interacted with the TCR without inducing a signal (1). Analogous results have been obtained by others showing that differential T cell signals may be obtained with mutated antigenic peptides (5). Together these data indicate that TCRs make a novel interaction with antagonist peptide–MHC complexes, which does not produce a typical signal. However, it is still unclear exactly what quantitative and qualitative differences distinguish the TCR interaction with agonist versus antagonist peptides. Furthermore, what signals (if any) result from antagonist–MHC interaction and how these translate into the T cell inhibition observed is unknown. Previous reports have focused on helper cell proliferation (1, 5), but other steps in the signaling pathway have not so far been analyzed for their sensitivity to antagonism.

We wished to investigate the nature of the TCR interaction with antagonist peptides and to explore the mechanism by which TCR antagonism inhibits T cell function. This report describes a large panel of TCR antagonists that inhibit CTL lysis in a clone-specific manner. Sensitivity to antagonist interaction was also analyzed for other T cell responses ranging from immediate to late activation events.

Materials and Methods

Cell Lines. The ovalbumin$_{257-264}$ (OVAp)[1]-specific, K^b-restricted CTL clones B3 and GA4 were derived and maintained as described (6) with weekly restimulation on the OVA-transfected EL4 clone E.G7 (7) in the presence of 5% rat Con A supernatant. TG-1 is a CTL clone derived from an OVAp/K^b-specific TCR transgenic mouse, which will be fully described elsewhere (F. R. Carbone, unpublished data). It uses the TCR $V_\beta 5$ chain expressed by B3 and a TCR $V_\alpha 2$ chain that pairs naturally with this β chain but does not derive from B3 (J. Kelley and F. R. Carbone, unpublished data). The VSV-1 CTL are specific for the VSV nucleoprotein (VSV-N), restricted by K^b, and were derived and maintained as described (8). EL4 was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and antibiotics (RP10 medium). The cell line FDC.P1 (9), a kind gift of Dr. Kevin Leslie

[1] *Abbreviations used in this paper:* OVAp, ovalbumin$_{257-264}$; VSV-N, vesicular stomatitis virus nucleoprotein.

(University of British Columbia, Vancouver, Canada), responds strongly to IL-3 and GM-CSF (10). This line was maintained in IL-3-containing tissue culture supernatants.

Peptides. Single amino acid variants of OVAp were initially made using the Multipin Synthesis System (11) (Chiron Mimotopes, Clayton, Australia). Some peptides were prepared using f-*moc* synthesis on a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) at the Howard Hughes Chemical Synthesis facility (University of Washington) or on a RaMPS apparatus (DuPont-NEN Products, Boston, MA), and were purified by HPLC (Waters, Milford, MA). Peptide concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL).

Prepulse CTL Lysis Assays. EL4 cells were labeled with ^{51}Cr-sodium chromate in RP10 for 1 h at 37°C at a concentration of 5×10^6/ml. A suboptimal concentration of OVAp or VSV-N peptide (3–10 pM) was introduced for the duration of the labeling. The cells were then washed three times in RP10. The cells were resuspended to 2×10^5 cells/ml, and 50 μl (10^4 cells) was transferred to a well of a round-bottomed 96-well plate containing 50 μl PBS or the test peptide diluted in PBS. In the initial screening the OVAp variant peptides were tested at ~4 μM concentration. After incubation for 30 min at 37°C, CTL were added in 100 μl RP10 to give an E/T ratio of 3:1. The assay plate was maintained in a 37°C incubator with 5% CO_2 for 2.5–5 h, after which the plate was centrifuged and 100 μl of the supernatant removed and counted.

Percent specific lysis was determined as follows: percent specific lysis = 100× [(experimental release − spontaneous release)/(detergent release − spontaneous release)]. Spontaneous release was <20% in all assays described. Percentage inhibition of lysis was calculated as follows: percent inhibition = $100\times[(A - B)/(A)]$, where A is the percent specific lysis in the absence of inhibitor, and B is the percent lysis in its presence.

In experiments to determine whether the variant peptides were agonists, the labeling was performed in the absence of added OVAp peptide. In some experiments, the OVAp-pulsed ^{51}Cr-labeled EL4 cells were washed three times and then pulsed with a second peptide for 1 h at 37°C and at 5×10^6 cells/ml in RP10. After a further three washes, the cells were diluted and incubated with the CTL as described above.

Cold Target Competition Assay. Hot targets were EL4, ^{51}Cr labeled and prepulsed with 3 pM OVAp as described above. Cold targets were unlabeled EL4, unpulsed or pulsed with OVAp (10 nM) or the D7 peptide (5 or 50 μM) as indicated. All target cells were washed three times in RP10 before addition to the assay. Hot targets were introduced at 10^4 cells/well, cold targets at 2×10^5/well, and CTL were added to 3×10^4/well to give a total volume of 200 μl. The CTL assay was then incubated and harvested as described above.

Serine Esterase Assay. EL4 cells were irradiated (20,000 rad) and then coated at the indicated concentrations of OVAp for 1 h at 37°C. The cells were washed three times and then incubated in RP10 media alone or with 1 μM D7 for a further 1 h at 37°C. After three washes, 2×10^5 pulsed EL4 in 100 μl were added to wells of a 96-well flat-bottomed plate containing B3 CTL (10^5/100 μl). Spontaneous release was measured from B3 cells incubated alone, while total serine esterase activity was determined from B3 cells treated with 0.5% NP-40 at the start of the assay. The plate was placed in a 37°C incubator with 5% CO_2 for 3–12 h. The supernatant was then assayed for BLT serine esterase activity, as described (12, 13). Briefly, the plate was centrifuged (5 min) and 20 μl of supernatant removed and mixed with 180 μl of reaction buffer (2×10^{-4} M N^{α}-benzyloxycarbonyl-L-lysine thiobenzyl ester and 1.1×10^{-4} M 5, 5′-dithio-bis-[2-nitrobenzoic acid] in PBS, pH 7.4 [Sigma Chemical Co., St. Louis, MO]). After incubation at room temperature for 1 h, the absorbance at 405 nm was measured in an ELISA plate reader (EL312e microplate reader; Bio-Tek Instruments, Winooski, VT). Data are expressed as percentage serine esterase release following the formula used for CTL lysis assays.

IL-3 Production. Culture conditions for this assay were identical to those used for the serine esterase assay, except that half the number of EL4 cells were used. After a 24-h incubation the plate was centrifuged briefly and 100 μl of supernatant was removed and diluted serially. The GM-CSF/IL-3-dependent line FDC.P1 (9, 10) was washed in RP10 and added to the diluted supernatants at 2×10^4 cells/well, giving a final volume of 200 μl. After 48 h, 0.5 μCi of [^{3}H]thymidine was added, the cultures were harvested 16 h later, and the ^{3}H incorporation was measured.

Ca^{2+} Flux Assay. EL4 cells were pulsed with OVAp at the indicated concentrations in RP3 (as for RP10 but with 3% FCS) for 45 min at 37°C, washed two times, and then incubated for a further 45 min in RP3 with or without 1 μM D7 peptide. After three washes, the EL4 cells were resuspended in ~100 μl RP3. B3 CTL were loaded for 90 min in Indo-1 (~25 μg/ml; Molecular Probes, Inc., Eugene, OR) at 37°C in RP3, washed twice, and resuspended at 10^6/ml in RP3. After warming to 37°C, 10^6 CTL were mixed with 3×10^6 EL4 cells in a total volume of 1.1 ml. It was found that under these conditions calcium flux in the CTL was undetectable until the cells were centrifuged together (data not shown). The cells were analyzed on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA) using Becton Dickinson software. The B3 cells were live gated on the basis of their Indo-1 loading. A baseline measurement for the ratio of calcium bound/unbound Indo (expressed as violet/blue ratio) in the B3 cells was then monitored for 1 min. The sample was then removed, and the cells were pelleted together briefly (~10 s) in a microfuge. The cells were immediately resuspended and analyzed on the FACStar Plus® over ~6 min. The data were reanalyzed using the software Reproman (D. Coder, Cell Analysis Facility, Department of Immunology, University of Washington) and M-Time (14). An analysis gate using forward and side scatter to exclude dead cells and tumor cells was applied.

Results

Variants at TCR Contact Sites of an Antigenic Peptide Are Clone-Specific Antagonists for CTL Lysis. TCR antagonists have so far only been described for the proliferative function of class II–restricted CD4$^+$ T cells (1, 5). Therefore, we wished to determine first whether the effects of antagonists could be extended to class I–restricted CTL, using target cell lysis as an assay. Previously we described the fine antigen specificity of two CTL clones, B3 and GA4, specific for the OVA peptide, OVAp (sequence SIINFEKL), in the context of K^b (6, 15, 16). In that analysis we proposed TCR contact residues at positions 4, 6, and 7 of the peptide, with position 2 contributing weakly to T cell recognition (16). These assignments correspond with the TCR contacts predicted from the crystal structure of OVAp/K^b (M. Matsumura and I. Wilson, personal communications). Accordingly, we generated 64 single amino acid variants of OVAp at these four positions (all 20 amino acids, except C, M, and W). These peptides were tested for their capacity to inhibit CTL lysis in an assay designed to distinguish TCR antagonism from competition for MHC binding. This involves prepulsing the target cells with a suboptimal dose of OVAp peptide before incubating them with the variant peptide and CTL. Such an assay is represented in Fig. 1, showing the effect of three OVAp variants on lysis by B3, GA4, and CTL specific for

an unrelated K^b-restricted peptide, VSV-N_{52-59} (sequence, RGYVYQGL) (8, 17). Another peptide is also described in this figure, V-OVA, which is comprised of three dominant TCR contacts from the OVAp peptide (positions 4, 6, and 7) incorporated into the VSV-N peptide. The OVAp variant D7 is a potent antagonist for both OVAp/K^b-specific CTL clones. Other peptides showed differential effects on the two OVAp-specific CTL clones: the peptide L6 antagonizes the lysis mediated by B3 but does not affect GA4. Conversely, V-OVA inhibits cytotoxicity by GA4 but not B3 (Fig. 1). Some peptides antagonized lysis by both CTL clones but with different efficiency, e.g., the peptide A4 is a strong antagonist of GA4 and a weaker antagonist of B3. These antagonists were all active at very low concentrations (100 pM to 1 nM). The existence of peptides that are antagonists for one OVAp/K^b-specific CTL but not the other demonstrates these variants do not simply catalyze displacement of bound OVAp peptide from the K^b groove. This conclusion is also supported by the inability of an unrelated K^b binding peptide (e.g., VSV-N) to decrease B3- or GA4-mediated lysis in these assays (data not shown).

In contrast to the dramatic effects on killing by B3 and GA4, none of the OVAp variants significantly inhibited lysis by the VSV/K^b-specific CTL, VSV-1 (Fig. 1 *c*). On the other hand, all the variants bound K^b molecules similarly to the OVAp peptide, as measured by a K^b stabilization on the mutant cell RMA-S (16, 18, 19, and data not shown). Taken together, these results suggest that the OVAp-specific CTL inhibition was not some form of MHC blockade or CTL toxicity.

In this way we analyzed the panel of OVAp variants for their capacity to cause a significant reduction in the lysis mediated by B3, GA4, and a third CTL clone, TG-1. The clone TG-1 derives from a OVAp/K^b-specific TCR transgenic mouse. The transgenes it expresses are the TCR β chain from B3, and a TCR α chain that is unrelated to that used by B3 (15, and F. R. Carbone, unpublished data). The results of this analysis are listed in Table 1. Of 65 variants tested, 26 (40%) were potent antagonists for one or more of the three CTL clones tested. Table 1 also describes the properties of these variants as antigens (agonists) for CTL lysis, since several peptides were agonists for one clone while antagonists for another. Some peptides were agonists at high concentrations and potent antagonists for the same clone at greater dilutions. These are identified as scoring both as agonist and antagonist. This result suggests that Table 1 probably underestimates the true number of antagonist OVAp variants, since most variants were initially tested at a single concentration ($\sim$4 μM, and hence agonists at this dose may be antagonists at other concentrations.

The majority of antagonists were found in variants at positions 4, 6, and 7, which we had previously shown to be critical TCR contacts (16). A very large proportion (13/16) of position 4 variants were antagonists. In contrast, only one variant at position 2 was a strong antagonist for one of the CTL clones, B3. In line with this, we had previously shown that alanine substitution at position 2 affected recognition by B3 but not by GA4 (16). Thus, the same positions of the peptide that are important for antigen recognition are involved in antagonist peptide recognition. In keeping with

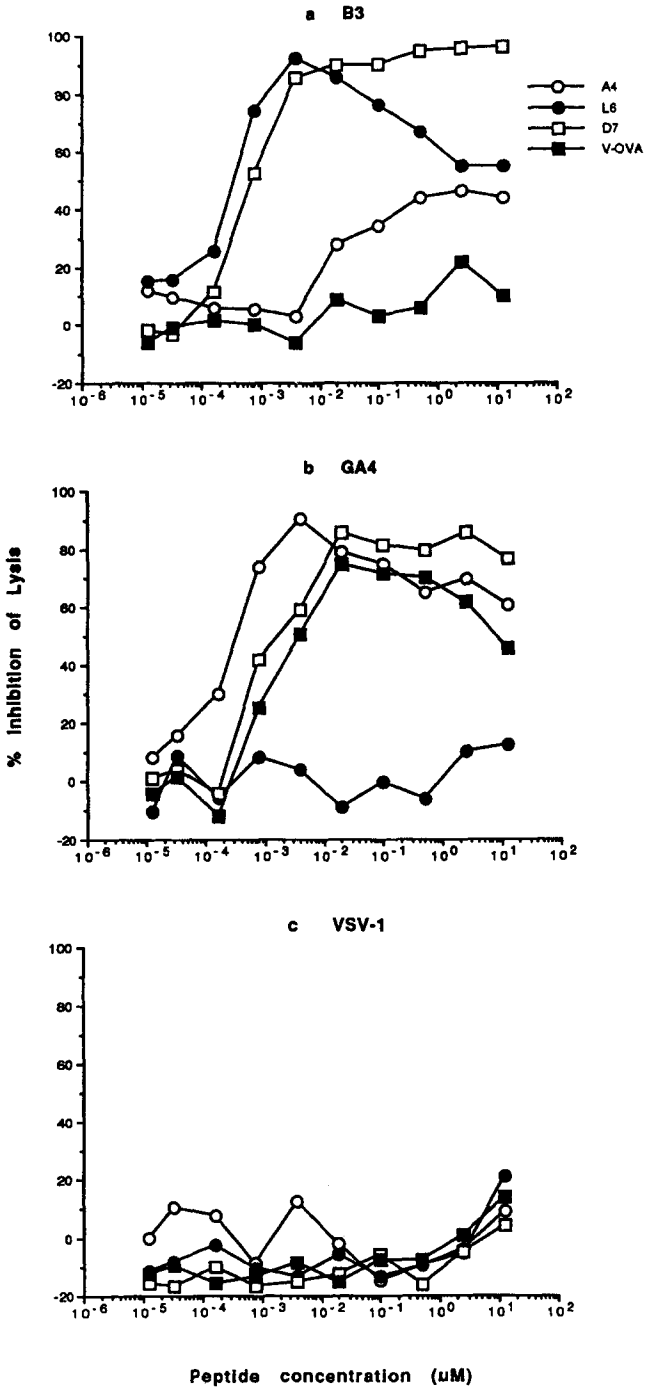

Figure 1. Variants of the OVAp peptide are clone-specific antagonists for CTL lysis. ^{51}Cr-labeled EL4 cells were prepulsed with 3 pM OVAp (*a* and *b*) or 3 pM VSV-N_{52-59} (*c*). The target cells were incubated with the indicated concentration of OVAp variant peptides. The following CTL were then added: (*a*) B3, (*b*) GA4, and (*c*) VSV-1. Lysis was determined 2.5–4 h later. Specific lysis by all CTL in the absence of added OVAp variant peptides was 25–30%. Data are shown as percent inhibition of this lysis, calculated as described in Materials and Methods. The sequences of the OVAp variants named are given in Table 1.

Table 1. *Variants of OVAp that Act as Antagonists for the OVAp/K^b-specific CTL Clones B3, GA4, and TG-1*

	Residues changed from $OVA_{257-264}$								B3		GA4		TG-1	
Peptide	S	I	I	N	F	E	K	L	Agonist	Antagonist	Agonist	Antagonist	Agonist	Antagonist
F2	•	F	•	•	•	•	•	•	−	+ +	+ +	+	+ +	−
A4	•	•	•	A	•	•	•	•	−	+	+	+ +	+ +	+
D4				D					−	+ +	+ +	+	−	+ +
E4				E					−	−	−	−	−	+ +
F4				F					−	−	−	+ +	+	+ +
G4				G					−	+ +	−	+ +	+ +	+ +
I4				I					−	+	−	+ +	+	+ +
L4				L					−	+ +	−	+ +	+ +	+ +
Q4				Q					−	+	−	+ +	+ +	−
R4				R					−	−	+	+ +	−	+ +
S4				S					+	+ +	−	+ +	+	+ +
T4				T					−	+ +	−	+ +	+ +	−
V4				V					−	+	−	+ +	+ +	+
Y4				Y					+	−	−	+ +	+ +	+ +
F6	•	•	•	•	•	F	•	•	+ +	+	+	−	−	+ +
I6						I			−	+ +	+	−	−	+ +
L6						L			+	+ +	−	−	+ +	+ +
P6						P			+	+ +	+ +	−	+	+ +
V6						V			−	+ +	−	+	−	+ +
Y6						Y			+ +	+	+	−	+	+ +
D7	•	•	•	•	•	•	D	•	−	+ +	−	+ +	+	+ +
E7							E		+ +	+	−	+ +	+	+ +
I7							I		+	+ +	+ +	+ +	+ +	+ +
L7							L		+	+ +	+ +	+	+ +	+ +
P7							P		−	+ +	+ +	−	−	+ +
V-OVA	R	G	Y	•	Y	•	•	•	−	−	+	+ +	−	+ +

Peptide names follow from the amino acid substitutions that distinguish them from OVAp, the sequence of which is given. The maximum capacity of these peptides to agonize or antagonize CTL-mediated lysis over a large concentration range (from ~1 μM to ~100 pM) is summarized. For agonists: −, <5% specific lysis; +, 5–30%; and + +, >30%. As antagonists: −, <20% inhibition of lysis; +, 20–50%; and + +, >50% inhibition.

this conclusion, there is pronounced clone specificity in susceptibility to antagonism. Indeed, when the degree of agonism and/or antagonism was evaluated (Table 1), none of the OVAp variants acted in the same way for all three CTL clones. The B3 and GA4 clones, which use different TCR V_β and V_α segments (J. Kelley and F. R. Carbone, unpublished data), shared the same pattern of antagonism for only 6 of the 26 peptides identified. Furthermore, the B3 and TG-1 clones, which have identical β chains, also overlap for only 6 of 26 peptides. Indeed, some peptides were antagonists for one clone while acting as agonists for another (Table 1). Together these data indicate there is TCR fine specificity for the interaction with antagonist peptides, probably involving similar TCR elements as are used for antigen recognition.

The Effect of Antagonists Is Not Mediated by CTL-CTL Interaction. In the assays described above, the antagonist peptides were present throughout the course of the CTL lysis assay. Since the CTL themselves express the appropriate restriction element (K^b), it was possible that the decreased lysis observed arose from CTL-CTL interaction, leading to an inhibition of function. Such effects have been reported previously including experiments using OVAp-specific CTL clones (20–22). In particular there is precedent for inhibition of CTL activation by interaction with CD8-bearing target cells (23). To test for this directly, we pulsed the antagonist peptide D7 onto either the target cells or the CTL before the assay. The data shown in Fig. 2 clearly indicate that while coating the effector cells in a high dose of the D7 peptide had no effect

on their subsequent lytic capacity, pulsing the target cells with this peptide profoundly inhibited lysis. Since the same protocol was used for pulsing the D7 peptide on both target and CTL, the result is probably not due to antagonist carryover into the assay. This phenomenon was CTL clone specific since when the peptide V-OVA was used in place of D7 to coat target cells, lysis by GA4 but not B3 was inhibited (data not shown). These data argue that antagonism involves recognition of antagonist peptide expressed on the APC rather than through CTL-CTL interaction.

Evidence for Direct TCR Recognition of Antagonist–MHC Complexes. Among the OVAp variants, we found some that acted as both agonist and antagonist for the same clone. This is demonstrated in Fig. 3 with the peptide P6. An agonist for the clone GA4, this peptide acts as both a weak agonist and an antagonist for B3. Thus at the same concentration, this peptide is capable of weakly stimulating B3 while antagonizing a stronger response. Several other peptides with similar activities were identified. The agonist properties of the OVAp variants analyzed are therefore listed in Table 1. These properties are characteristic of partial agonist/antagonists, a class of ligand defined as having low efficacy, i.e., producing a weak maximal response (24). Thus these data indicate that the TCR interacts with the partial agonist/antagonist producing a suboptimal lytic signal. Other variants were strict antagonists; i.e., they were unable to stimulate CTL lysis at any concentration tested. We tested TCR recognition of one of these peptides (D7) using a cold target competition assay (see Materials and Methods). As shown in Fig. 4, lysis of ^{51}Cr-labeled target cells by B3 was not inhibited by unlabeled EL4 but was efficiently inhibited by EL4 coated in OVAp peptide. When D7 peptide–coated EL4 cells were used as competitors, lysis was reduced by ~50%. The data are presented for cold targets pulsed with 5 and 50 μM D7, which gave essentially identical results. A similar degree of inhibition was observed when the cold targets were pulsed at 1 μM D7 and with cold/hot target ratios as low as 7.5:1 (data not shown). The effect titrated out at lower cold/hot

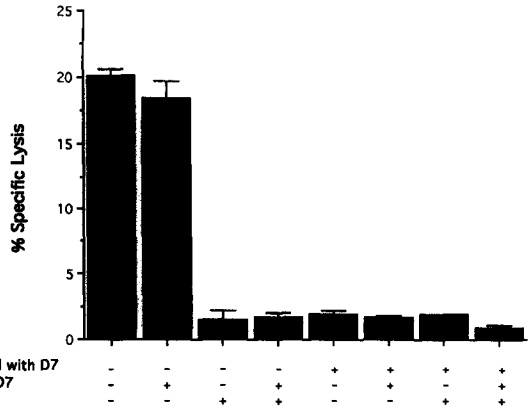

Figure 2. TCR antagonism is not mediated through T cell–T cell contact. A prepulse lysis assay was performed as described in Fig. 1, except that either the OVAp-coated EL4 target cells or the B3 effector cells were then pulsed with 5 μM of the D7 antagonist peptide for 1 h before the assay, as indicated. In some cases, free D7 peptide (100 nM) was added directly to the assay, as indicated.

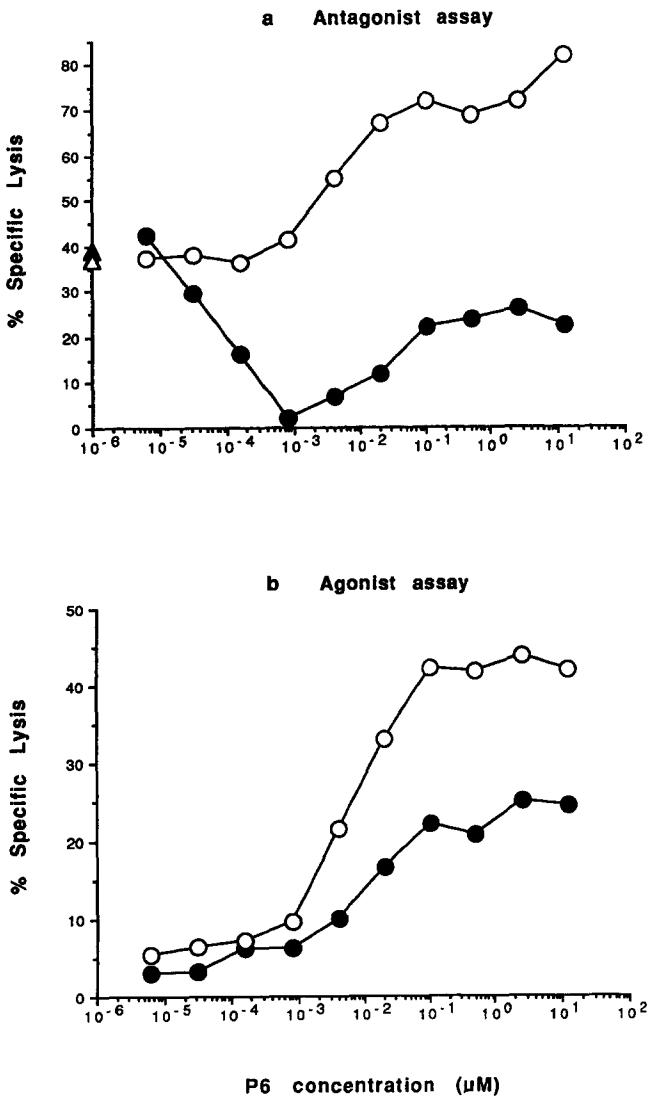

Figure 3. The P6 peptide is a partial agonist/antagonist for the B3 CTL and is an agonist for GA4. The CTL clones B3 and GA4 were tested for their response to the peptide P6 as an antagonist (*a*) or an agonist (*b*). EL4 were prepulsed with 3 pM OVAp (*a*) or media alone (*b*) before incubation with the indicated concentrations of P6. The lysis by B3 (●) and GA4 (○) is indicated by circles. Lysis in the absence of added P6 peptide is represented in *a* by triangles.

ratios (4:1) (data not shown), which explains why no inhibition of CTL lysis was observed in Fig. 3 (the B3 CTL themselves could act as cold targets, but in those experiments they are at an effective cold/hot ratio of only 3:1). These data suggest that there is a direct interaction between the B3 TCR and the D7 peptide–K^b complex, although this interaction does not lead to lysis of the target cell.

Serine Esterase Release, Cytokine Production, and Calcium Flux Are All Susceptible to TCR Antagonism, but Differ in the Degree of Sensitivity. The assays described so far assess the effect of antagonists on a single aspect of CTL activation, i.e., lysis. Since the mechanism of TCR antagonism is unknown, it is valuable to determine which events in the T cell signaling

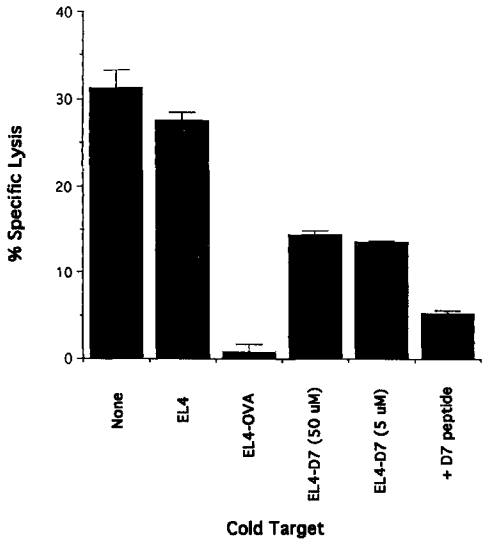

Figure 4. Cold target competition assay demonstrates a weak direct interaction between TCR and antagonist–MHC complex. ^{51}Cr-labeled EL4 cells were prepulsed with 3 pM OVAp. Cold targets were EL4 cells that were pulsed with OVAp or D7 peptides as indicated. The effect of D7 peptide added directly to the assay (100 nM) is identified (+ D7 peptide). The hot/cold target ratio was 1:20 and the effector/hot target ratio was 3:1.

cascade are affected by antagonists and which are not. We first tested another parameter that may be related to the cytotoxic function, serine esterase release (12, 13). As shown in Fig. 5 *a*, the D7 peptide itself did not stimulate serine esterase release, but it did drastically inhibit the release in response to various doses of OVAp peptide coated onto stimulator cells. This effect was consistent over a long time course: serine esterase assays from 3 to 24 h after initiation of the culture showed similar effects (data not shown). We also assayed the production of lymphokines after CTL activation. Production of both GM-CSF and IL-3 has been reported for $CD8^+$ cells (10, 25, 26). Hence we tested supernatants from CTL cultures 24 h after stimulation for their capacity to stimulate the FDC.P1 cell line, which responds strongly to both cytokines (9, 10). A similar antigen sensitivity was observed for this function as for lysis and serine esterase release (Fig. 5 *b*). Profound inhibition of cytokine production was observed when stimulators were pulsed with antagonist peptides. Thus the inhibitory effect of the antagonists is long lasting and affects several aspects of the T cell response. Both assays depicted in Fig. 5 also show that the effects of the antagonists are, to some degree, surmountable through using higher doses of antigen in the prepulse (compare inhibition at 3 pM OVAp with that at 9 pM). Lysis of target cells is also surmountable in this way (data not shown).

A very early event in T cell activation is mobilization of intracellular calcium. We tested the effect of TCR antagonists on this response. The assay shown in Fig. 6 *a* shows a strong Ca^{2+} flux response of the B3 CTL clone to EL4 cells coated in a high concentration of OVAp (1 μM). Similar responses were observed when anti-CD3 crosslinking was used to stimulate the B3 cells (data not shown). In contrast, no Ca^{2+} response was stimulated by EL4 alone or EL4

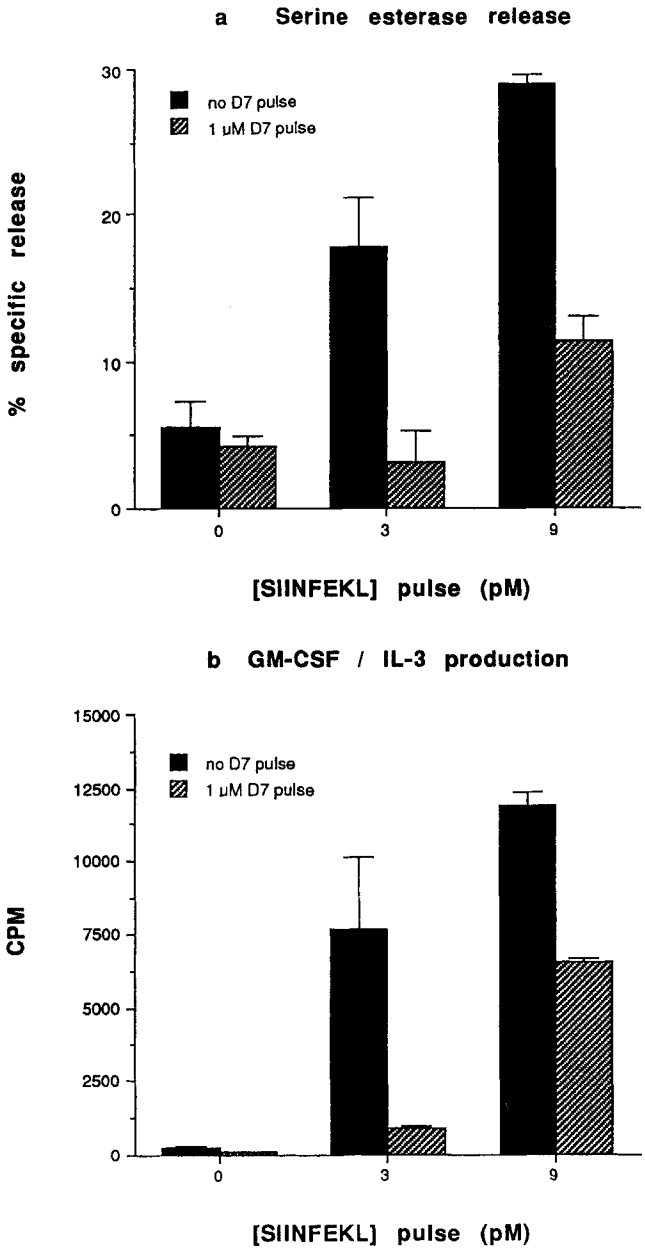

Figure 5. Serine esterase release and GM-CSF/IL-3 production are susceptible to TCR antagonism. (*a*) B3 cells were cultured with irradiated EL4 stimulator cells pulsed with the indicated concentration of OVAp peptide. Some stimulators were pulsed for a second time with D7 peptide (1 μM), as indicated. Serine esterase release was determined after 12 h. (*b*) Culture supernatants after CTL stimulation with the indicated stimulator cells were diluted 1:5 and assayed for stimulation of FDC.P1 cell proliferation, measured by [^{3}H]thymidine incorporation. This cell responds to both GM-CSF and IL3 (10). Both assays were conducted in triplicate and error bars show the standard deviations.

coated in 1 μM D7 (Fig. 6 *a*). As was the case in the other assays described, a suboptimal calcium flux response of B3 was observed when stimulators were pulsed in picomolar concentrations of OVAp. We therefore assayed calcium flux in response to EL4 cells coated with various low doses of OVAp, with or without subsequent pulsing with the D7 antagonist

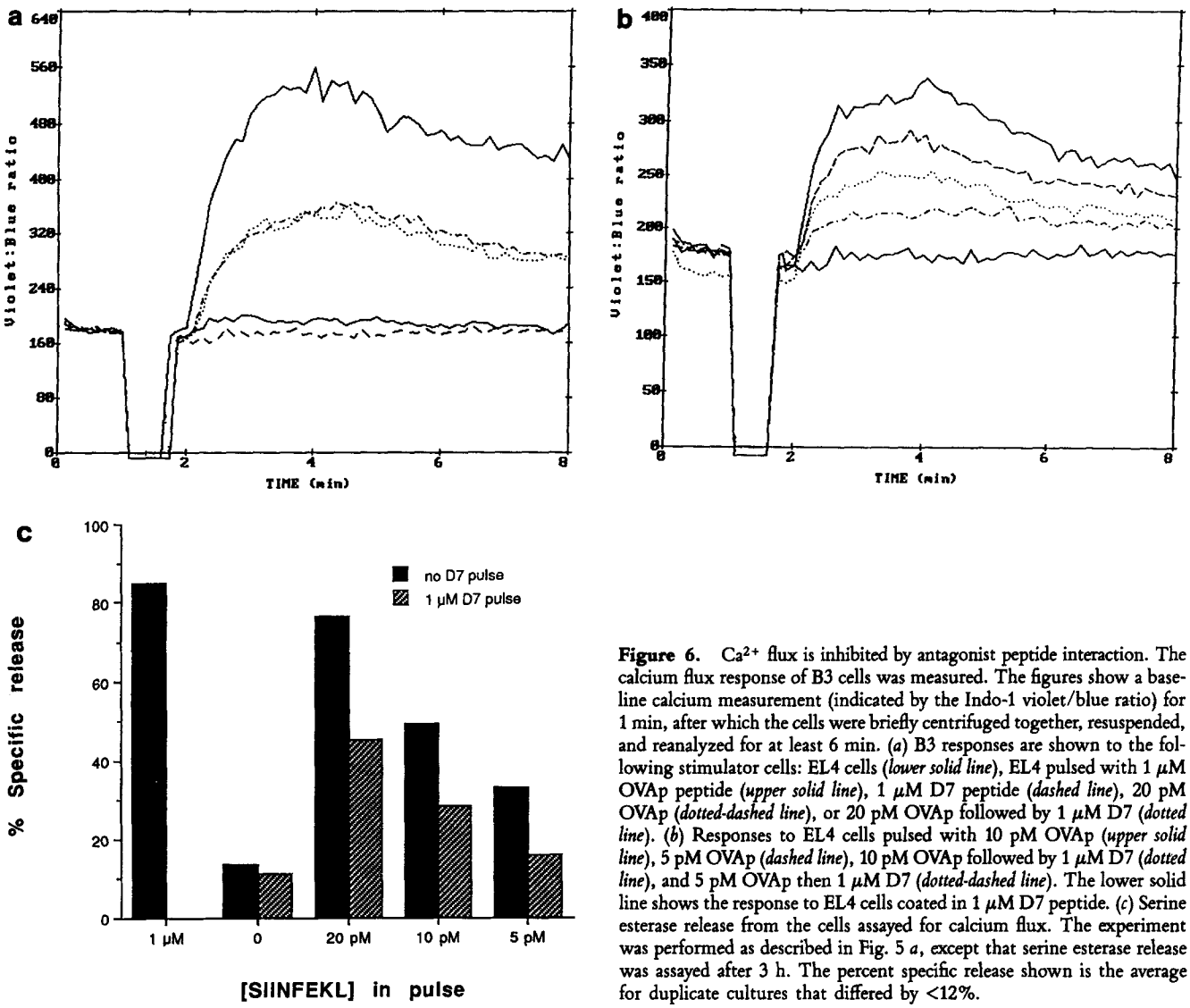

Figure 6. Ca^{2+} flux is inhibited by antagonist peptide interaction. The calcium flux response of B3 cells was measured. The figures show a baseline calcium measurement (indicated by the Indo-1 violet/blue ratio) for 1 min, after which the cells were briefly centrifuged together, resuspended, and reanalyzed for at least 6 min. (*a*) B3 responses are shown to the following stimulator cells: EL4 cells (*lower solid line*), EL4 pulsed with 1 μM OVAp peptide (*upper solid line*), 1 μM D7 peptide (*dashed line*), 20 pM OVAp (*dotted-dashed line*), or 20 pM OVAp followed by 1 μM D7 (*dotted line*). (*b*) Responses to EL4 cells pulsed with 10 pM OVAp (*upper solid line*), 5 pM OVAp (*dashed line*), 10 pM OVAp followed by 1 μM D7 (*dotted line*), and 5 pM OVAp then 1 μM D7 (*dotted-dashed line*). The lower solid line shows the response to EL4 cells coated in 1 μM D7 peptide. (*c*) Serine esterase release from the cells assayed for calcium flux. The experiment was performed as described in Fig. 5 *a*, except that serine esterase release was assayed after 3 h. The percent specific release shown is the average for duplicate cultures that differed by <12%.

peptide. Immediately after the flux assay, the remaining cells were incubated for 3 h and serine esterase release was determined, so that this aspect of activation could be directly compared to the flux response. The calcium flux response to EL4 cells coated at 20 pM OVAp is shown in Fig. 6 *a*. This response was not affected when D7 was pulsed onto the stimulator cells (Fig. 6 *a*). In contrast, the serine esterase response of these same cells was clearly inhibited by D7 pulsing (Fig. 6 *c*), indicating a differential sensitivity between these two responses. Antagonism of the calcium flux response was observed, however, when lower doses of antigen were used for pulsing. OVAp pulsing at doses of 10 and 5 pM leads to decreased flux responses (Fig. 6 *b*) (note the reduced scale relative to Fig. 6 *a*). Under these conditions, pulsing with the D7 peptide leads to strong inhibition of the calcium flux response (Fig. 6 *b*) and serine esterase response (Fig. 6 *c*). Yet here also, some difference was seen in the degree of antagonism between the two assays: at 5-pM pulsing the serine esterase assay is almost fully inhibited (Fig. 6 *c*), whereas calcium flux is only reduced by ~60% (Fig. 6 *b*).

Together, these data suggest that the effect of antagonist peptides is evident at the level of Ca^{2+} flux, but that this event is more resistant to antagonism than downstream responses.

Discussion

We demonstrate here the existence of TCR antagonist peptides affecting CTL function. We generated a large panel of variants of the peptide OVAp that acted as antagonists for three OVAp/K^b-specific CTL clones. Several aspects of these results are interesting.

(*a*) The peptide residues identified as important for TCR recognition (positions 4, 6, and 7) were also the positions that yielded multiple antagonists, whereas substitutions at a position that was less important for TCR recognition (position 2) produced only one potent antagonist peptide. This indicates that the recognition of antagonist peptide probably involves the same portions of the TCR as are used to recognize the antigenic peptide (16).

(*b*) No simple chemical features unite the antagonist variants at a certain position. Generally, aliphatic residues acted as antagonists for at least one CTL clone, but occasional negatively charged and aromatic residues also emerged as potent antagonists, especially at position 4 (Table 1). One striking trend, however, was that negatively charged residues (Asp and Glu) at position 7 of OVAp were strong antagonists for GA4. In contrast, it has been demonstrated that among variants at this position the best agonists have positively charged residues (His, Lys, or Arg) (15). In this case then, there may be charge interactions between the peptide and the TCR and/or K^b molecule that dictate whether the T cell interaction will lead to stimulation or antagonism. However, the variants at position 4, which act as GA4 antagonists, are so varied that it seems most likely that the effect is due to a loss of interaction with asparagine at this position, rather than a novel interaction with the substituted amino acid. Our data thus do not support the contention by Alexander et al. (27) that antagonists arise primarily from conservative substitutions at TCR contacts.

(*c*) Over one-third of the single amino acid OVAp variants tested were antagonists for one or another CTL clone. Looking at position 4, this proportion rises to >80% of tested variants. Thus the frequency and diversity of antagonist variants are strikingly high. We also analyzed a peptide (V-OVA) that differs more extensively from OVAp. Although it shares only four of eight residues with OVAp, this peptide is a potent antagonist for the clone GA4. Together, these results indicate that peptides that are quite distantly related to the antigen may be antagonists. This conclusion suggests that specific antagonists could be frequent among MHC binding peptides and hence may exist among naturally occurring self-peptides. Furthermore, the amounts of antagonist peptide needed for detection in our assays (20–200 fmol for several variants) indicate physiological levels of natural peptides (28, 29) could influence CTL function. Thus we propose to use the high sensitivity of our assays to search for such naturally occurring antagonist peptides.

(*d*) All of the antagonists identified had differential effects on the three CTL clones tested. This ranged from opposites in stimulatory properties (agonist vs. antagonist) to more subtle differences in the degree of the T cell response. This result has important ramifications on the potential use of antagonists in inhibiting T cell responses in vivo since it is clear, based on our observations, that a polyclonal T cell response will be difficult to antagonize. Indeed, we have observed such resistance to antagonism in the response of a bulk OVA/K^b-specific CTL line (our unpublished observations). On the other hand, T cell responses of less diversity, as have been reported in several cases (30–36), may be very susceptible to antagonism.

Another characteristic of TCR antagonists is that they inhibit T cell function in MHC competition assays with greater efficiency than expected from their MHC binding capacity (1). In our system, MHC binding competition only causes inhibition of lysis when unrelated K^b binding peptides are present at at least 1,000-fold higher concentration than targeting peptide (data not shown). However, lower doses of antagonist peptides are needed to show inhibition in this assay. Thus, variants A4 and P6 are equally effective in K^b blockade for VSV-N_{52-59}-specific CTL, but P6 is at least 10-fold more efficient than A4 in inhibition of B3 response to OVAp, correlating with their properties as B3 antagonists (data not shown).

It was previously unclear what sort of interaction occurred between the TCR and the antagonist–MHC complex. Indeed, previous studies failed to detect any form of T cell stimulation through antagonist–peptide interaction (1). We present two lines of evidence indicating direct TCR recognition of the antagonist complex. First we describe a large class of TCR partial agonist/antagonists. At particular concentrations some of these peptides are weak stimulators, but at the same time act as antagonists (see Fig. 3). Thus, there is clearly a specific TCR interaction involving peptides that act as antagonists. Second, we investigated the activity of a pure antagonist in cold target competition assays. The inhibition observed with antagonist peptide-coated cold target cells supports the idea that TCR-mediated engagement is occurring, effectively distracting the CTL from lysing the OVAp-coated hot targets. It is not clear exactly why the inhibition was consistently low (~50%), despite titrations of antagonist peptide and cold/hot target ratios, but this result may indicate a weak TCR interaction with the antagonist–MHC.

Taken together, these data can be interpreted as meaning there is a low affinity interaction between TCR and antagonist–MHC that is sufficient to occupy TCR but that falls short of stimulating the necessary numbers of TCR for triggering a response. This view was also concluded in a recent paper from Sette's group (27). However, an equally valid interpretation is that antagonism operates through a qualitative difference in the signal imparted through agonist vs. antagonist peptide. This could be due to conformational changes induced in the TCR itself or result from differences in the activation of other costimulatory molecules or adhesion molecules (37–39). A few examples of differential signaling induced by variant antigens have been described (5, 40). By this argument, the weak interaction between the T cell and antagonist-coated APC (this paper) would result from the lack of (conventional) T cell stimulation, since TCR activation is associated with both enhanced T cell–APC conjugation and potentiation of accessory molecule activity (37–39). In this scenario, our data do not directly address the affinity between TCR and antagonist peptide. Direct measurements of the affinity of TCR for antagonist–MHC complexes (41, 42) will thus be valuable in approaching this issue. However, our observations with partial agonist/antagonists can be interpreted as indirect support for a qualitative difference in the T cell response. For example, the response of B3 to high concentrations of the peptide P6 is not altered by the simultaneous presence of a suboptimal dose of OVAp (Fig. 3, *a* vs. *b*). This indicates that the response to OVAp is fully antagonized at this concentration of P6 and that the weak agonist response seen is due entirely to inefficient signaling through P6 recognition. This scheme most easily fits in with a model in which efficient engagement of the TCR by P6 results in an inefficient signal for target cell lysis.

We have begun an analysis of several T cell functions for their susceptibility to antagonism. The release of serine esterase from cytotoxic granules, and the synthesis of GM-CSF and/or IL-3, were found to be antagonized in a similar way to that observed for CTL lysis. Also studied was the Ca^{2+}

flux response, an early event. This was also inhibited by an antagonist peptide, but was more resistant to antagonism than another parameter of activation, serine esterase release. The possibility that assay of calcium flux reflects a nonphysiological degree of stimulation is unlikely since the flux response and the other responses studied showed remarkably similar antigen dose responses. Thus, these data indicate that sequential stages in the activation pathway may show a differential sensitivity to antagonist interactions.

Last, it is interesting to note the possible relevance of antagonist peptides to T cell development. During positive selection, thymocytes interact with self-peptide–MHC complexes on epithelial cells, resulting in their rescue from programmed cell death (43–47). On the other hand, it is evident that engagement with stimulatory antigen–MHC complex at this stage leads to T cell tolerance by deletion or anergy (46, 48). Thus the nature of the TCR–ligand interaction leading to positive selection is presently an unresolved paradox. However, the characteristics of the positive selection interaction are strikingly similar to those we describe here for TCR antagonists: a clone-specific, MHC-restricted TCR interaction evidently occurs (43–46) but does not appear to induce activation (47). In this paper we have described TCR antagonists for the CTL clone TG-1, which derives from a TCR transgenic mouse strain (F. R. Carbone, unpublished data). Hence, we will use this system to assess the role of TCR antagonists in T cell positive selection.

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Address correspondence to M. J. Bevan, Howard Hughes Medical Institute, Department of Immunology, SL-15, University of Washington, Seattle, WA 98195.

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