population eruptions in the northwestern Atlantic, there were great difficulties in finding an underlying cause (5). We have added the possibility of another "cause," namely, that population eruptions may be an underlying feature of the dynamics without any change in physical or biological conditions.

Efforts to detect density dependence are likely to fail precisely for those species with strong density-dependent recruitment (*22–24*) if they are spatially distributed in the fashion we have examined here. We have shown that long transient behavior is more likely to result when local density dependence is strong rather than weak. Thus, ironically, problems in detection of density dependence are most likely to occur for those species for which the underlying density-dependent mechanisms are in fact the strongest. Just this effect has been observed in studies of the viburnum whitefly (*24*).

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Sequential Interactions of the TCR with Two Distinct Cytoplasmic Tyrosine Kinases

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The T cell antigen receptor (TCR) initiates signals by interacting with cytoplasmic protein tyrosine kinases (PTKs) through a 17-residue sequence motif [called the antigen recognition activation motif (ARAM)] that is contained in the TCRζ and CD3 chains. TCR stimulation induces the tyrosine phosphorylation of several cellular substrates, including the ARAMs. Lck kinase activity is required for phosphorylation of two conserved tyrosine residues in an ARAM. This phosphorylation leads to the recruitment of a second cytoplasmic PTK, ZAP-70, through both of the ZAP-70 Src homology 2 domains and its phosphorylation. Thus, TCR signal transduction is initiated by the sequential interaction of two PTKs with TCR ARAMs.

The multisubunit TCR is composed of the TCRαβ heterodimer, a ζ family homo- or heterodimer (ζ-ζ, ζ-η, or ζ-γ), and the CD3 chains (γ, δ, and ε) (*1*). With the use of chimeric receptors, the signal transduction functions of the TCR have been localized to a common motif, here called the ARAM, that contains the sequence $YXXLX_{(6-8)}$ YXXL (*2*) and is present in the cytoplasmic domains of ζ and each of the CD3 chains (*3–6*). One of the earliest events associated with TCR signal transduction is tyrosine phosphorylation of cellular proteins that include the ARAMs of the TCRζ and CD3 chains (*4, 5, 7*). Three PTKs have been implicated in TCR signal transduction: Lck, Fyn, and ZAP-70. Lck is not generally found directly associated with the TCR but interacts with the coreceptors CD4 and CD8, which colocalize with the TCR during antigen recognition (*8*). Studies of Lck-deficient cells indicate that Lck is essential for TCR signal transduction, including tyrosine phosphoprotein induction (*9, 10*). Fyn associates, albeit at low stoichiometry, with the TCRζ chain (*11*). Studies of Fyn-deficient mice reveal a TCR signal transduction defect in the most mature thymocyte subsets, but mature peripheral T cells are not as affected (*12*). After TCR stimulation, the cytoplasmic PTK ZAP-70 rapidly associates with the ζ and CD3 chains and undergoes tyrosine phosphorylation (*4, 13–15*). A 72-kD tyrosine phosphoprotein, presumably the related PTK Syk (*16*), associates with membrane immunoglobulin (Ig) on B cells and with the IgE Fc receptor on mast cells (*17*). Studies of chimeric transmembrane proteins that have included ZAP-70, Syk, Lck, or Fyn suggest that ZAP-70 or Syk can regulate the function of downstream signal transduction molecules (*18*).

The mechanism by which the TCR interacts with these distinct families of PTKs is not understood. Studies have shown that ZAP-70 associated only with tyrosine-phosphorylated ζ (*14*). In addition, the association of ZAP-70 with the CD8-ζ chimera in a COS cell that stably expresses a CD8-ζ chimera (COS-18) requires the co-expression of either Lck or Fyn with ZAP-70 (*14, 19*). In J.CaM1.6, a Jurkat-derived mutant line (*20*), TCR stimulation does not increase tyrosine phosphoproteins or activate the phosphatidylinositol pathway because of the loss of functional Lck (*10, 20*). TCR stimulation of Jurkat cells, but not of J.CaM1.6, resulted in ζ phosphorylation (Fig. 1A, upper panel) although both cells have equivalent amounts of ZAP-70, TCR, and ζ chain protein (Fig. 1B) (*20, 21*). Unlike Jurkat cells, neither a phosphorylated nor an unphosphorylated form of ZAP-70 associated with ζ in TCR-stimulated J.CaM1.6 cells (Fig. 1A, upper and lower panels, respectively). These results provide genetic evidence that Lck function is required for tyrosine phosphorylation of ζ and for the recruitment and tyrosine phosphorylation of ZAP-70.

The requirement for Lck was further analyzed in COS-18 cells. As we have already shown (*14, 19*), cotransfection of Lck with ZAP-70 results in the association of ZAP-70 with CD8-ζ, as well as in the tyrosine phosphorylation of ZAP-70 and CD8-ζ (Fig. 2). However, a kinase-inac-

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tive Lck mutant that had a mutation of Lys^{273} to Ala [Lck(A273)] (*22*) could not substitute for the wild-type Lck. In contrast, a mutation in the phosphotyrosine-binding site of the Src homology 2 (SH2) domain of Lck [Arg^{154} to Lys; Lck(K154)] (*22, 23*) had no effect on the association of ZAP-70 with CD8-ζ or on the phosphorylation of CD8-ζ or ZAP-70. Thus, Lck kinase activity, but not its SH2 domain, is required for CD8-ζ tyrosine phosphorylation and ZAP-70–CD8-ζ association in COS cells. These data, however, do not exclude a role for the Lck SH2 domain in T cell activation, as has been shown (*24*).

The preferred association of ZAP-70 with the tyrosine-phosphorylated form of ζ (*14*) suggested that ZAP-70 may be recruited to the ζ chain through its SH2 domains. The two SH2 domains contained in ZAP-70, here called SH2(N) and SH2(C), have divergent sequences (*14*). Mutation at each phosphotyrosine-binding site (*23*) of either the SH2(N) (Arg^{37} to Lys; K37) or the SH2(C) (Arg^{190} to Lys; K190) domain (*25*) eliminated the ability of ZAP-70 to associate with the CD8-ζ chimera in the presence of Lck (Fig. 3). Moreover, both SH2 mutants were tyrosine-phosphorylated to a lesser extent than wild-type ZAP-70 (*21*). Therefore, the function of both SH2 domains is necessary for the stable binding of ZAP-70 to ζ sequences. These observations are consistent with a study with fusion proteins encompassing both SH2 domains of ZAP-70 (*26*). This suggests that the affinity of each SH2 domain with its target is low and that the stable association of ZAP-70 with phosphorylated ζ requires both ZAP-70 SH2 domains and two separate phosphotyrosine residues.

To determine the structural features in the phosphorylated ζ chain required for the ZAP-70 interaction, we used four synthetic peptides encompassing the ζ ARAM closest to the NH_2-terminus (Fig. 4A). We could not detect binding of ZAP-70 to the unphosphorylated peptide (M1) or to peptides singly phosphorylated at either tyrosine residue (P1 or P2) (Fig. 4B). However, ZAP-70 bound, in a dose-dependent manner, to the doubly phosphorylated P1,2 peptide. An equimolar mixture of P1 and P2 (P1 + P2) did not bind ZAP-70, which suggests that ZAP-70 binds only to an individual doubly phosphorylated ARAM. Stimulation of the TCR did not influence the ability of ZAP-70 to bind to the peptides, and the P1,2 peptide bound unphosphorylated and phosphorylated ZAP-70 equally well (*21*). Neither phospholipase Cγ1

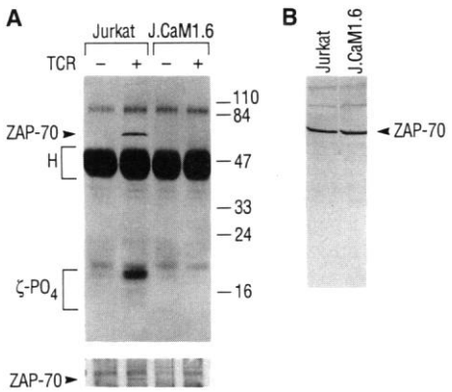

Fig. 1. The association of ZAP-70 with ζ is dependent on the expression of Lck. (**A**) The ζ chain was immunoprecipitated from unstimulated (–) or TCR-stimulated (+) Jurkat cells (*35*). The immunoprecipitates were analyzed by immunoblotting with a mAb to phosphotyrosine, 4G10 (upper panel), or polyclonal antiserum to ZAP-70 (lower panel). The positions of ZAP-70, the immunoprecipitating antiserum heavy chain (H), phosphorylated ζ (ζ-PO_4), and molecular size standards (at right in kilodaltons) are shown. (**B**) We immunoblotted total cell lysates from each cell line with an antiserum to ZAP-70 to quantitate the amount of expression of ZAP-70 protein.

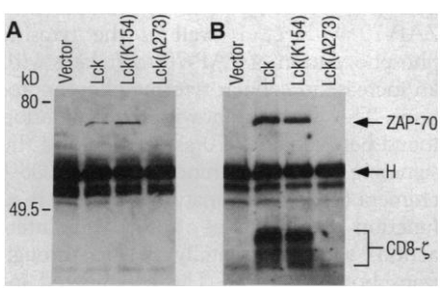

Fig. 2. Loss of Lck kinase activity, but not SH2 function, abrogates the Lck-dependent induction of ZAP-70–CD8-ζ association. The ZAP-70 construct epitope-labeled on its COOH-terminus (*22*) was transfected into COS-18 cells with vector, wild-type Lck, SH2 mutant Lck(K154), or kinase-inactive mutant Lck(A273) as indicated (*22*). The CD8-ζ chimera was immunoprecipitated with a mAb to CD8 (OKT8). The immunoblot was analyzed with a mAb to the epitope (**A**) or a mAb to phosphotyrosine (**B**). Lck(A273) had impaired kinase activity (*21*). The Lck(K154) mutation corresponds to a critical residue in the phosphotyrosyl-binding pocket of the Lck SH2 domain (*23*). Markers are indicated as in Fig. 1.

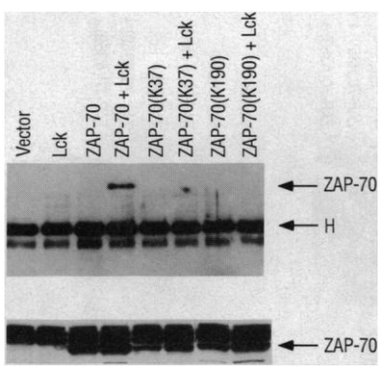

Fig. 3. Impairment of ZAP-70–CD8-ζ association by the mutation of either of the ZAP-70 SH2 domains. The indicated forms of ZAP-70 [wild-type ZAP-70, SH2(N) mutant (K37), and SH2(C) mutant (K190)], epitope-labeled on their COOH-termini, were transfected into COS-18 cells in the presence or absence of Lck (*22, 25*). CD8-ζ was immunoprecipitated with OKT8 and analyzed by immunoblotting with a mAb to the epitope (upper panel). For each SH2 mutation, the Arg contained in the FLVRE (*2*) sequence involved in phosphotyrosyl binding was changed to Lys (*23, 25*). We analyzed total cell lysates from the same transfectants by immunoblotting with a mAb to the epitope to quantitate the amount of ZAP-70 expression (lower panel). The upper two bands represent nonspecific reactivity of the mAb with cellular proteins.

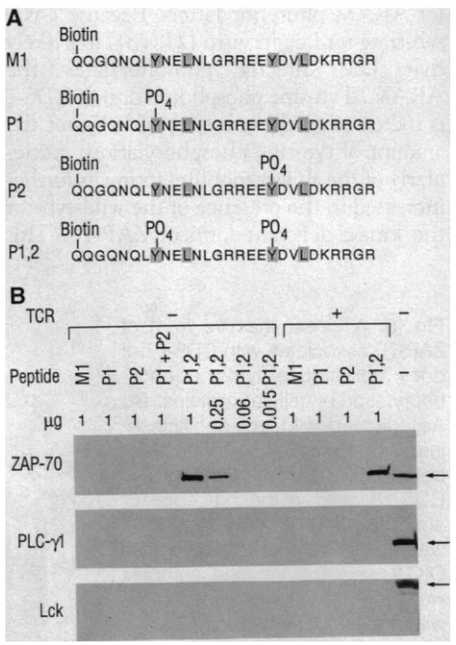

Fig. 4. Binding of ZAP-70 to a peptide encompassing a doubly tyrosine-phosphorylated ARAM. (**A**) Amino acid sequence of peptides used here (*2, 36*). Tyrosine and leucine residues of the ARAM are shaded in each line. M1 is unphosphorylated; P1 is phosphorylated (PO_4) at the more NH_2-terminal Tyr; P2 is phosphorylated at the more COOH-terminal Tyr; and P1,2 is phosphorylated at both places. The binding site of biotin is indicated. (**B**) Total cell lysates from unstimulated (–) or TCR-stimulated (+) Jurkat cells were mixed with the indicated peptides. The amount of peptide is shown in micrograms. Peptides were isolated with avidin-conjugated beads, and the complexes were analyzed by immunoblot analysis with the antibodies shown at left. The binding of ZAP-70 from 5.0×10^7 cells could be detected with as low a concentration as 120 nM (0.25 μg) but not 30 nM (0.06 μg) P1,2. The lane at right represents the total cell lysate and demonstrates the integrity of the blotting antibodies (arrows).

(PLCγ1) nor Lck bound the singly or doubly phosphorylated peptides (Fig. 4B), and no other tyrosine phosphoproteins were detected bound to doubly phosphorylated peptides (*21*). In addition, a fusion protein with both SH2 domains of ZAP-70 expressed in bacteria bound to the doubly phosphorylated peptide (*21*), suggesting that ZAP-70 binds directly to the phosphorylated ARAM.

To determine if the kinase activity of ZAP-70 is required for ARAM phosphorylation, we transfected wild-type or the kinase-inactive form of ZAP-70 either alone, or together with Lck, into COS-18 cells (Fig. 5, A and B). Both the wild-type and the kinase-inactive mutant of ZAP-70 (Lys^{369} to Ala; A369) (*27*) coprecipitated with the CD8-ζ chimera in a Lck-dependent manner. This observation, together with data in Fig. 2, demonstrates that the kinase activity of Lck, but not ZAP-70, is required for ARAM phosphorylation. Because ζ is a substrate for Lck in vitro (*21, 28*), it is likely that Lck directly phosphorylates the ARAM. Tyrosine phosphorylation of CD8-ζ is increased in the presence of Lck, but the amount of tyrosine phosphorylation, particularly of the slower mobility forms, is further increased in the presence of the wild-type or the kinase-deficient form of ZAP-70. This latter effect may be due to the protection of phosphotyrosine residues from cellular protein tyrosine phosphatases by the ZAP-70 SH2 domains, similar to that observed in other systems (*29*).

The CD8-ζ–associated wild-type or kinase-inactive ZAP-70 molecules were tyrosine-phosphorylated equivalently. Thus, the phosphorylation of ZAP-70 does not require its own kinase function, although the sites of phosphorylation in the kinase-inactive form may differ from those in the wild type. Such phosphorylation could occur directly between Lck and ZAP-70 or could occur after the recruitment of ZAP-70 to ζ. To test these hypotheses, we expressed Lck and ZAP-70 in COS-7 cells, which do not express CD8-ζ. Expression of ZAP-70 alone results in a small amount of basal tyrosine phosphorylation of ZAP-70 (Fig. 5C). In the presence of Lck, there was a small increase in tyrosine phosphorylation of ZAP-70. However, tyrosine phosphorylation of ZAP-70 increased with co-expression of Lck and the CD8-ζ chimera, which suggests that ZAP-70 phosphorylation is facilitated by the recruitment of ZAP-70 to phosphorylated ζ.

One proposed function of the kinase domain of ZAP-70 is the tyrosine phosphorylation and activation of downstream molecules involved in TCR signal transduction (*14, 18, 19*). We have reported (*14*), and confirm here, that an increase in cellular tyrosine phosphoproteins was observed in COS-18 cells cotransfected with Lck and wild-type ZAP-70. However, a reduction was observed in cells cotransfected with Lck and the kinase-inactive mutant of ZAP-70 (Fig. 5D). A similar reduction was found for each of the ZAP-70 SH2 mutants (*21*). These results indicate that the kinase activity of ZAP-70 contributes to the induction of cellular phosphoproteins after its recruitment to ζ.

Our studies demonstrate that the TCR interacts with two classes of PTKs in a sequential manner. The initial interaction involves ARAM tyrosine phosphorylation and is dependent on Lck kinase activity. During antigen recognition, the CD4 or CD8 coreceptors colocalize with the TCR by binding to major histocompatibility complex (MHC) molecules (*8*). Because CD4 and CD8 stably associate with Lck, they could deliver Lck into close proximity with ARAMs and induce ARAM phosphorylation. Coreceptor colocalization with the TCR by the use of monoclonal antibodies (mAbs) increases ζ phosphorylation and other manifestations of TCR signal transduction (*30*). However, on the basis of studies of J.CaM1.6, Lck dependence in TCR signal transduction does not require coreceptor function, suggesting that Lck can also directly interact with the TCR to phosphorylate the ARAMs (*10*). Recruitment of ZAP-70 to phosphorylated ζ facilitates ZAP-70 phosphorylation, which is likely to be regulated by Lck as well. In some T cells, Fyn may provide the functions mediated by Lck (*11, 12, 31*). The association of a ZAP-70 with ζ, as well as the tyrosine phosphorylation of ZAP-70, correlates with an increase in cellular tyrosine phosphoproteins. This is consistent with the correlation found between ZAP-70 association and the signaling function of truncations of a CD8-ζ chimera (*4*). An explanation is that ZAP-70 function is activated as a result of its interaction with Src family PTKs through transphosphorylation. The coordinated interaction between the TCR and cytoplasmic PTKs may provide a paradigm for the interactions of other ARAM-containing receptors with cytoplasmic PTKs.

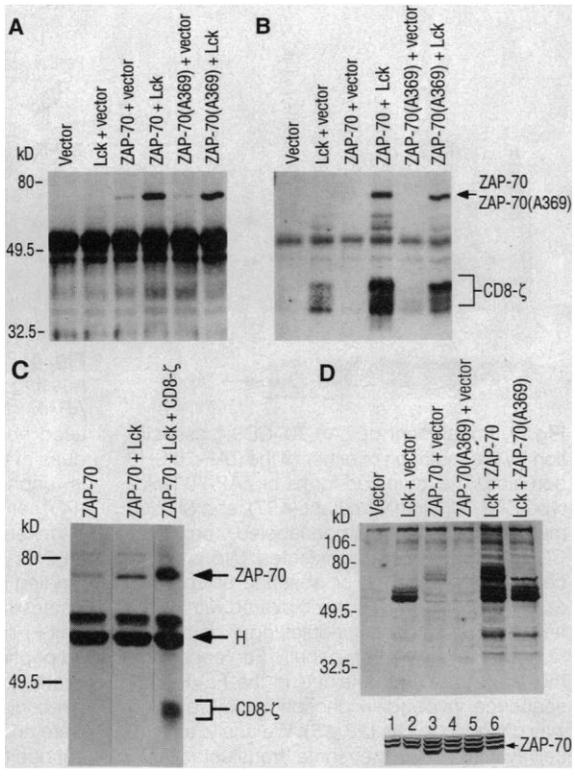

Fig. 5. A kinase-inactive form of ZAP-70 associates with CD8-ζ but does not induce tyrosine phosphorylation of cellular proteins. (**A**) As indicated, wild-type and kinase-inactive forms of NH_2-terminal labeled ZAP-70 were expressed in COS-18 cells in the presence or absence of Lck (*22, 27*). CD8-ζ was immunoprecipitated with OKT8, and the immune complex was analyzed by immunoblotting with a mAb to the epitope (A) or to phosphotyrosine (**B**). The mutant ZAP-70 essentially had no kinase activity (*21*). (**C**) COS-7 cells were transfected with the indicated combinations of constructs expressing Lck, ZAP-70, and CD8-ζ. ZAP-70 was labeled with an epitope at its COOH-terminus. From each transfectant, ZAP-70 was immunoprecipitated with a mAb to the epitope and analyzed by immunoblotting with a mAb to phosphotyrosine. We reblotted the same filter with a mAb to the epitope to confirm that the amount of ZAP-70 protein in each lane was equivalent (*21*). (**D**) COS-18 cells were transfected with the same constructs as described in (A). Total cell lysates were analyzed by immunoblotting with a mAb to phosphotyrosine. The amount of ZAP-70 expression in each transfectant was detected with a mAb to epitope and is shown in the lower panel. The two upper bands in the lower panel are derived from COS cells because they are also detected in cells transfected only with vector.

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22. COS-7 (derived from monkey kidney) and its CD8-ζ chimera stable transfectant (COS-18) have been described (*14*). These cell lines were transfected with 2 μg of each construct with DEAE-dextran (*14*). The total amount of DNA for each transfection was equalized with vector DNA. Cells were harvested 48 hours after transfection. Equivalent expression amounts of mutants and of wild-type Lck and ZAP-70 were confirmed by immunoblotting. Lck and its mutants were expressed with the pSM vector (*32*). The kinase mutant of murine Lck, Lck(A273), was created by two-step polymerase chain reaction (PCR). First, two PCR products were made from the combination of the following primers: (A) a 5′ primer containing a Bam HI site at nucleotide position 502 [J. D. Marth, R. Peet, E. G. Krebs, R. M. Perlmutter, *Cell* **43**, 393 (1985)] and a 3′ primer containing a mutation of Lys^{273} to Ala and (B) a 5′ primer corresponding to the 3′ primer of (A) and a 3′ primer containing a Pst I site at nucleotide position 940. Second, with a 5′ primer of (A) and a 3′ primer of (B), the Bam HI–Pst I fragment containing the Lys to Ala mutation was created from the mixture of PCR fragments of (A) and (B). This fragment replaced the corresponding region in wild-type murine Lck. The SH2 mutant of Lck, Lck(K154), was created by the replacement of the Bam HI–Nco I fragment of wild-type Lck with the PCR product from the 5′ primer containing the mutation of Arg^{154} to Lys and a 3′ primer containing a Nco I site at nucleotide position 745. The epitope derived from influenza hemagglutinin (*33*) was introduced at the Apa I site 3′ of the ZAP-70 kinase domain for COOH-terminal labeling. 12CA5 is a mAb against the epitope (*33*). Rabbit antisera and a mAb to Lck were used as indicated. OKT8 is a murine mAb to the CD8α chain.
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35. Jurkat, the human leukemic T cell line, and its Lck kinase–deficient derivative J.CaM1.6 (*20*) were stimulated with C305, a mAb to TCR (*34*), as described (*13*). Methods for SDS–polyacrylamide gel electrophoresis, immunoblotting, and immunoprecipitation have been described [B. Irving and A. Weiss, *Cell* **64**, 891 (1991)]. 4G10 is a murine mAb to phosphotyrosine (Upstate Biological, Lake Placid, NY). Rabbit antiserum was generated against a ZAP-70 synthetic peptide (*14*). 387 is a rabbit antiserum to ζ [D. Orloff *et al.*, *J. Biol. Chem.* **264**, 14812 (1989)].
36. Peptides were synthesized with a peptide synthesizer (Applied Biosystems, Foster City, CA). For the precipitation with peptides, cell lysates were mixed with biotinylated peptides for 1.5 hours. We added avidin-conjugated agarose beads to this mixture to isolate the peptide complexes. After 1.5 hours of incubation, the precipitated samples were washed and analyzed by immunoblotting procedures. The mAb to ZAP-70 was produced against recombinant ZAP-70 protein encompassing SH2 domains and is specifically reactive with ZAP-70 but not Syk.
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T Cell Deletion in High Antigen Dose Therapy of Autoimmune Encephalomyelitis

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Encounters with antigen can stimulate T cells to become activated and proliferate, become nonresponsive to antigen, or to die. T cell death was shown to be a physiological response to interleukin-2–stimulated cell cycling and T cell receptor reengagement at high antigen doses. This feedback regulatory mechanism attenuates the immune response by deleting a portion of newly dividing, antigen-reactive T cells. This mechanism deleted autoreactive T cells and abrogated the clinical and pathological signs of autoimmune encephalomyelitis in mice after repetitive administration of myelin basic protein.

Despite the role of acquired immunity in mounting a defensive reaction against infectious agents, it is known that high doses of antigen can paradoxically suppress immune responses in adult animals (*1–3*). This type of antigen-specific tolerance, termed high dose suppression or high zone tolerance, involves extrathymic mechanisms in mature T lymphocytes (*4, 5*). In other experimental settings, mature T lymphocytes have been shown to die after T cell receptor (TCR) stimulation by processes that may involve the Fas antigen, interferon-γ or T cell growth lymphokines, and cytolytic mechanisms, but it is not understood what role these processes play in high dose suppression (*6–11*). To better understand the paradox of high dose suppression, we studied its mechanism in two in vitro models: a $CD4^+$ T lymphocyte clone, A.E7, that is suppressed at high doses of a pigeon cytochrome c peptide containing amino acids 81 to 104 (PCC) (*12*) and primary lymph node T cells (LNTCs) from a mouse line that is transgenic for a TCR ($V_\alpha 2.3$, $V_\beta 8.2$) that recognizes myelin basic protein (MBP) peptide (Ac1–11) and confers susceptibility to experimental allergic encephalomyelitis (EAE) (*13*). EAE is an autoimmune disease model in which axon sheaths of the central nervous

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