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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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25. Both SH2(N) and SH2(C) mutants of ZAP-70 were created by a two-step PCR. For the SH2(N) mutant, the Sac II (at position 220)–Hae II (at position 394) wild-type fragment was replaced by a PCR fragment containing a mutation of Arg^{37} to Lys. For the SH2(C) mutant, the wild-type Kpn I (at position 695)–Sca I (at position 868) fragment was replaced by a PCR product containing a mutation of Arg^{190} to Lys.
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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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system are destroyed by infiltrating T cells that are specific for myelin protein components MBP or proteolipid protein (PLP) (*14–16*). This causes a relapsing paralysis similar to the human disorder multiple sclerosis (*14, 15*). We also examined the mechanism of high antigen dose treatment in EAE (*15*).

The proliferation of A.E7 cells was maximal at 0.01 μM PCC antigen and then increasingly suppressed with greater concentrations (Fig. 1A) (*17*). For $V_\alpha2.3^+$, $V_\beta8.2^+$ LNTCs, proliferation peaked at a higher antigen concentration (10 μM MBP), but further increases in antigen also suppressed proliferation (Fig. 1B). Quantitation of viable T cells after 72 hours in both systems revealed a reduction of more than 90% at high antigen doses compared with doses that gave maximal proliferation, which paralleled the [^{3}H]thymidine incorporation (Table 1). Cell cycle blockade could have accounted for decreased cell numbers, but the same fraction (70%) of viable A.E7 cells had entered S phase as indicated by bromodeoxyuridine (BrdU) incorporation at both high and low concentrations of antigen (Table 1). This contradictory lack of proliferation at high antigen doses despite cell cycle progression and interleukin-2 (IL-2) production (below) was explained by the microscopic observation of cell death by apoptosis in both A.E7 cells and LNTCs exposed to high or low concentrations of antigen, but not in samples without antigen (*18*).

To understand the mechanism of T cell death in high-zone tolerance, we examined the influence of IL-2. Both A.E7 cells and transgenic LNTCs produced maximal IL-2 at suppressive doses of antigen, in accord with our findings that early activation events, including IL-2 gene expression, proceed normally at high antigen doses (Fig. 1A) (*19*). To determine whether IL-2 produced early after stimulation took part in activation-induced T cell death at late times (*10, 11*), we studied the effects of IL-2 and antigen on the viable T cell recovery. LNTCs with the $V_\alpha2.3$, $V_\beta8.2$ TCR were activated with the Ac1–11 peptide for 48 hours, washed to remove the antigen, and then cultured in IL-2 (3, 12, 25, or 50 IU/ml) for an additional 48 hours (*20*) (Table 2). Incorporation of [^{3}H]thymidine at this time showed the expected correlation between cell cycle progression and IL-2 concentration. The IL-2–treated cells were then rechallenged with increasing doses of peptide antigen in the continued presence of IL-2. The loss of $V_\alpha2.3^+$, $V_\beta8.2^+$ T cells compared with the unstimulated controls was greater with higher antigen concentrations at all doses of IL-2. Moreover, cell loss at any dose of antigen was increased in direct proportion to the concentration of IL-2 used in the initial stimulation. Cells treated with IL-2 at 3 U/ml behaved like resting LNTCs: The $V_\beta8.2^+$ cells expanded at low but not high peptide amounts.

We next tested whether endogenously produced IL-2 was necessary for death. Because LNTCs die from lymphokine withdrawal (*11*), we used A.E7 cells stimulated with antigen (1 or 10 μM) in the presence of 3C7, a monoclonal antibody (mAb) that blocks the α chain of the IL-2 receptor, or mAb S4B6, which neutralizes IL-2 itself (*20*). Both mAbs protected A.E7 cells from antigen-induced death, but an IL-4–blocking mAb (11B11) did not (Table 2). Thus, activated T helper cell–type 1 (T_H1 cells) can make sufficient IL-2 to lead to their own demise.

These results raised the possibility that high doses of antigen could be used to

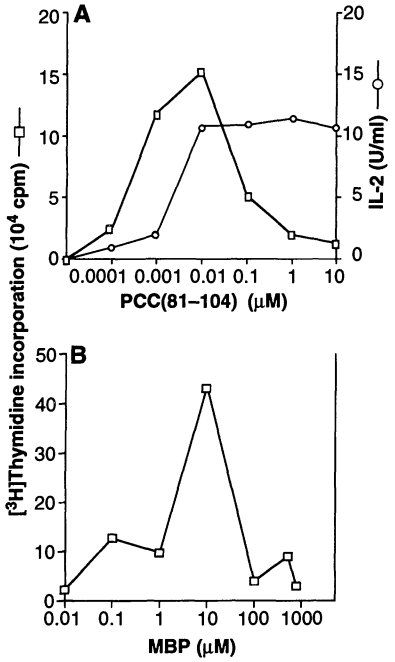

Fig. 1. High dose suppression in (**A**) A.E7 T cells and (**B**) $V_\alpha2^+$, $V_\beta8^+$ transgenic LNTCs. [^{3}H]Thymidine incorporation assay (in counts per minute) for proliferation (squares) and bioassay of IL-2 (circles). In a separate experiment with $V_\alpha2^+$, $V_\beta8^+$ transgenic LNTCs, production of IL-2 (per milliliter) was 1 U, 3 U, 9.4 U, and 11.8 U at 0.1, 10, 100, and 750 μM antigen, respectively. Data are representative of five experiments.

Table 1. Viable cell recovery after the stimulation of resting T lymphocytes with high doses of antigen. The number of CD4$^+$ T cells (in thousands) was quantitated by flow cytometry with antibodies to $V_\alpha11$ for A.E7 cells and antibodies to $V_\beta8.2$ for $V_\alpha2^+$, $V_\beta8^+$ LNTCs after stimulation (*10*). Doses of 100 and 750 μM were not tested in A.E7. In other experiments, stimulation of $V_\alpha2^+$, $V_\beta8^+$ LNTCs with antigen doses <10 μM showed gradual increases in cell number to a maximum between 1 and 10 μM.

Cells tested	Antigen stimulation (μM)						
	0	0.01	0.1	1	10	100	750
Resting A.E7	0.6 (11)*	4.4 (74)*	2.9 (73)*	0.4 (68)*	0.4 (73)*		
Resting $V_\alpha2^+$, $V_\beta8^+$ LNTCs	1.5				27.7	0.9	1.2

*In separate experiments, the percent of $V_\alpha11^+$ cells that incorporated BrdU in an 18-hour pulse (*10*) was measured.

Table 2. Role of IL-2–stimulated cell cycling in the loss of viable cells after antigen stimulation. The percent reduction of viable cells represents the cell number at indicated antigen dose divided by the cell number for 0 μM antigen sample for the same treatment group. The number of cells recovered for 0 μM (in thousands) were 16.4, 36.5, 87.7, and 83.4 for IL-2 at 3, 12, 25, and 50 IU/ml, respectively. Negative numbers indicate cell expansion.

Treatment		[^{3}H]Thymidine incorporation (cpm)	Percent reduction of viable cells after antigen (μM)		
IL-2 (U/ml)	mAb specificity		1	10	100
		*$V_\alpha2.3^+$, $V_\beta8.2^+$ LNTCs**			
3	–	4,985	−197	−28	79
12	–	41,651	30	57	92
25	–	70,038	41	83	93
50	–	115,578	78	90	95
		Resting A.E7 cells			
–	None	–	81	78	
–	Anti–IL-2R†	–	4	0	
–	Anti–IL-4	–	71	73	

*$V_\alpha2.3^+$, $V_\beta8.2^+$ LNTCs were activated and treated with the indicated doses of IL-2 for 48 hours and then rechallenged with antigen for an additional 48 hours in the continued presence of IL-2. Control experiments showed that the Ac1–11 peptide had no intrinsic toxicity on LNTCs (*24*). †Treatment with mAb S4B6 [antibody to IL-2 (anti–IL-2)] also prevented loss of A.E7 cells at 1 and 10 μM antigen.

specifically eliminate responding T cells in a disease setting. We tested whether repeated intravenous (iv) treatments with large amounts of soluble MBP could delete peripheral autoreactive T cells and thereby improve the course of EAE. We used an adoptive transfer model of EAE because it reproducibly results in severe disease and avoids thymic contribution to the encephalitogenic pool (*15*). Transgenic $V_\alpha2.3^+$, $V_\beta8.2^+$ T lymphocytes were activated with Ac1–11 peptide for 4 days in vitro, and then 3×10^7 cells were injected into matched (B10.PL or PL/J) naïve recipients (*13, 21*). Ten days later, the recipient B10.PL mice had a significant fraction of $V_\alpha2.3^+$, $V_\beta8.2^+$ cells among $CD4^+$ T lymphocytes in the spleen (23.7%, compared with 1.8% in mock transfer animals) and had severe progressive limb paralysis (disease grade 4) (Fig. 2A). In contrast, animals that received eight iv treatments of MBP (400 μg, twice daily on days 0, 2, 4, and 6) had an 84% deletion of $V_\alpha2.3^+$, $V_\beta8.2^+$ cells (3.8%) and no disease (Fig. 2B). Eight iv doses of the Ac1–11 peptide (1400 μg) also caused an 81% deletion of $V_\alpha2.3^+$, $V_\beta8.2^+$ cells (4.5%) and abrogated disease (Fig. 2C). Repeated injections of a control antigen (ovalbumin) had little effect on the amount of encephalitogenic cells or disease severity (disease grade 3) (Fig. 2D). The $CD4^+$ cells that expressed other TCRs were not deleted by MBP treatment (Fig. 2). In PL/J mice, untreated animals that received transgenic cells had 11.9% $V_\alpha2.3^+$, $V_\beta8.2^+$ cells among splenic $CD4^+$ T lymphocytes (Fig. 2F) compared with 2.9% in mock transfer mice (Fig. 2E), and repeated doses of MBP again caused significant deletion, to 3.8% $V_\alpha2.3^+$, $V_\beta8.2^+$ cells (Fig. 2H). A single dose of antigen caused much less cell loss (Fig. 2G). Control animals that received resting transgenic T cells and similar MBP treatments showed no deletion, consistent with our in vitro observation that cell cycling is required for cell loss (*11*). Thus, in two susceptible mouse strains, multiple iv doses of soluble nominal antigen deleted the activated encephalitogenic transgenic T cells with concomitant improvement of disease.

Because the donors and recipients in the transgenic experiments were not fully syngeneic, we also evaluated iv MBP treatments in EAE in syngeneic nontransgenic animals (*22*). LNTCs from MBP-primed (PL × SJL)F_1 mice that were activated in vitro with MBP caused severe disease in naïve recipients after 7 to 10 days (Fig. 3A) (*23*). Multiple iv injections of MBP (400 μg) abrogated clinical symptoms: The mean clinical score in untreated mice was 3.1, but in treated mice it was 0.1 ($P < 0.0005$) (Fig. 3A), and the disease incidence was decreased (100% in the untreated group; 20% in the treated group, but the onset of disease was delayed more than 35 days). The same antigen therapy together with 60,000 IU of IL-2 each day for the first 5 days after transfer also improved the disease: The mean clinical score in untreated mice was 3.2, but in treated mice it was 0.5 ($P < 0.0005$) (*24*), suggesting that anergy, which is reversed by IL-2, was not involved (*25*). In contrast, IL-2 treatment without antigen rechallenge was rapidly fatal (*24*). The majority of treated animals remained disease-free, although retransfer of encephalitogenic T cells into treated animals after three-and-a-half disease-free months caused a rapid onset of paralysis (*24*), implying the absence of long-lived suppressor cells. Histopathologically, animals that received encephalitogenic cells but no treatment exhibited destruction of the spinal cord architecture with extensive areas of demyelinated axons on a background of fibrillary astrogliosis and macrophages containing myelin debris compared with controls (Fig. 3B) (*18*). In mice treated with iv MBP, there was dramatic protection of the myelin sheaths from inflammation and immune damage, and in many areas of the spinal cord, there was essentially normal cytoarchitecture (Fig. 3B).

We also detected T cell deletion in nontransgenic animals by staining MBP-activated LNTCs before transfer with a vital membrane dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) that is measurable by flow cytometry (Fig. 3C) (*23*). Nine days after transfer $CD4^+$, DiI-stained cells were detected in the lymph nodes of untreated animals that developed severe hindlimb paralysis (disease grade 3). After the iv administration of MBP on days 0, 2, and 4 after transfer, abrogation of clinical signs of EAE was associated with an 80% reduction in $CD4^+$, DiI^+ cells. Similar doses of ovalbumin only slightly reduced the $CD4^+$, DiI^+ cells and disease severity which may be due to nonspecific lymphokine effects or competition for MHC presentation. Proliferation assays of T cells from treated animals revealed a decreased precursor frequency of MBP-specific cells, indicating functional tolerance (*23*).

We found that the paradoxical suppression of T cell responses by high antigen doses is due to apoptosis caused by large concentrations of IL-2 and antigen. This seems counterintuitive because one would expect that T cells should expand more vigorously (to provide better immune protection) if confronted with a large inoculum of a pathogen. However, we have shown that antigen responses are governed by an intrinsic biochemical feedback mechanism in T cells. We found that a sequence of three events leads to apoptosis: (i) T cell activation and growth lymphokine synthesis, (ii) cell cycle progression, and (iii) TCR reengagement causing apoptosis. Thus, T cells "sense" the intensity of an immune response by the amount of cell cycling. Further antigen stimulation then attenuates the immune response by decreasing the number of reactive

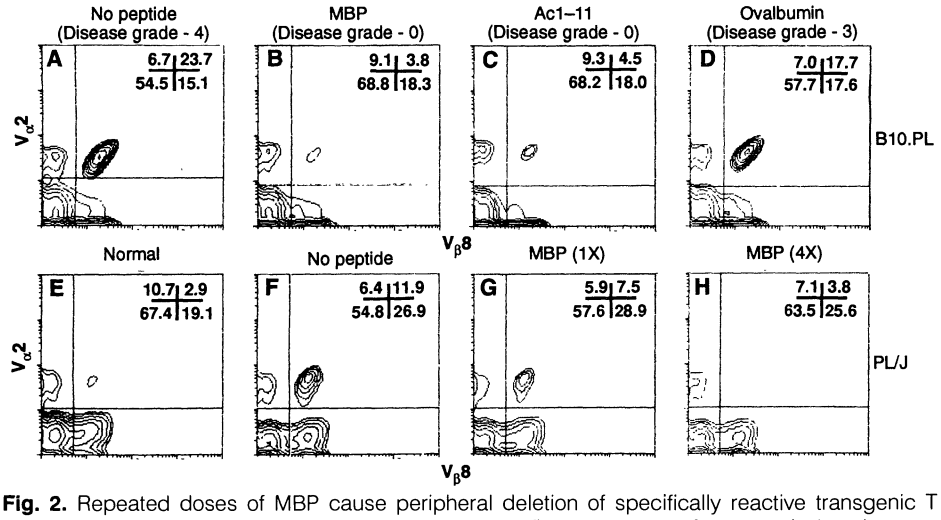

Fig. 2. Repeated doses of MBP cause peripheral deletion of specifically reactive transgenic T lymphocytes. Contour plots showing three-parameter flow cytometry of transgenic lymphocytes ($V_\alpha2^+$, $V_\beta8^+$) transferred into recipient mice. Splenocytes were gated on $CD4^+$ cells. Groups include B10.PL mice that were (**A**) untreated or treated intravenously with (**B**) whole MBP (400 μg), (**C**) Ac1–11 peptide (1.4 mg), or (**D**) ovalbumin (400 μg) twice daily on days 0, 2, and 4 after cell transfer. Test groups in PL/J mice received (**E**) no transgenic cells or received cells and were (**F**) untreated or intravenously treated with either (**G**) one injection of MBP (400 μg) on day 0 or (**H**) twice daily injections of MBP on days 0, 2, 4, and 6 after transfer. Populations of nonreactive TCRs ($V_\beta6$) showed no change in cell number. Disease was graded: 0, no symptoms; 1, limp tail; 2, moderate hindlimb weakness; 3, severe hindlimb weakness; 4, hindlimb paralysis; 5, whole body paralysis; and 6, death. Each flow cytometry plot is from an individual animal. Results are representative of three experiments.

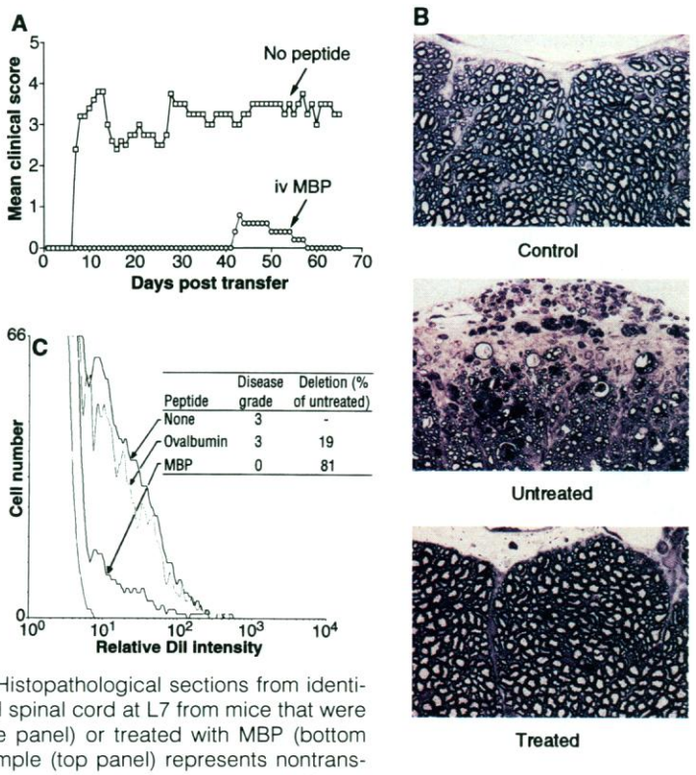

Fig. 3. Repeated injections of MBP abrogate EAE. (**A**) The mean clinical score of affected animals versus days after the transfer of encephalitogenic LNTCs into naïve recipient mice. The (PL × SJL)F_1 mice received MBP (400 μg) in PBS intravenously twice daily on days 0, 2, and 4. Disease was graded as in Fig. 2. Administration of low doses of MBP (100 μg) did not improve the health of diseased animals. Mean clinical score represents the average of disease grade for each group of five mice, and this experiment is representative of three experiments. (**B**) Histopathological sections from identical regions of the dorsal spinal cord at L7 from mice that were either untreated (middle panel) or treated with MBP (bottom panel) (*16*). Control sample (top panel) represents nontransferred, non–MBP-treated mice (magnification ×271). (**C**) Nontransgenic, MBP-reactive T lymphocytes are deleted by repeated MBP administration. Histograms showing detection of encephalitogenic LNTCs that before transfer were stained with the fluorescent vital membrane dye DiI. Live lymph nodes harvested from the various treatment groups were gated on $CD4^+$ cells (50,000 events) and analysis of DiI intensity is shown. Treatment groups include nontreated mice and mice administered either ovalbumin or MBP (400 μg) twice daily on days 0, 2, and 4 after transfer. The percent deletion is calculated as 1 − (cell number detected in treated/cell number detected in untreated). Results are representative of four experiments.

T cells and thereby self-regulates the production of IL-2 (and other lymphokines). We have called this feedback mechanism "propriocidal regulation" (*11*). In the present experiments, T cell death stabilizes IL-2 accumulation over a 1000-fold range of antigen (Fig. 1A). Because lymphokines are toxic in large doses, the propriocidal mechanism protects the organism from being injured by its own immune response (*26*). Thus, our results provide a biologically coherent explanation for the paradox of death at high antigen doses.

Our results shed light on recent observations that strongly immunostimulatory viruses delete responsive T cells and eliminate a recall response (*1–3, 7–9*). Our data imply that the stronger the initial proliferative response to antigen, the more vulnerable T cells are to apoptosis by antigen re-engagement. T cell stimulation can therefore be viewed as a balance between proliferation and death with the latter prevailing at high concentrations of lymphokine and antigen. Thus, effective vaccination and the establishment of T cell memory will involve not only clonal expansion but also the avoidance of T cell death. The mechanism we describe also explains the observation that a period of "rest" facilitates anamnestic responses, because allowing T cells to exit the cell cycle will reduce their susceptibility to apoptosis (*27*).

We found that antigen administration can be used to delete autoreactive T cells and protect myelin sheaths from immunological damage. This in vivo deletion adhered to the predictions made from our in vitro investigations and likely results from T cell apoptosis. The eradication of disease-causing T cells by iv antigen infusion has therapeutic potential. In a disease for which the antigen is known, such as myasthenia gravis, an antigen treatment that targets T cells for deletion is now testable. Thus, our studies provide a rational basis for antigen-specific therapy in T cell–dependent diseases.

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17. The A.E7 cells are a $CD4^+$ T_H1 clone stimulated by PCC(81–104) in the context of H-$2E^k$ (*12*). MBP-reactive lymph node cells were from $V_\alpha2$, $V_\beta8$ TCR transgenic mice. Proliferation assays used 1×10^4 cells per well with 5×10^5 syngeneic splenocytes [A.E7 used B10.A splenocytes given 30 Gy (1 Gy = 100 rads); MBP used untreated B10.PL splenocytes] (*13*). The addition of antigen-presenting cells (APCs) reduced the antigen concentration at which peak proliferation of $V_\alpha2^+$, $V_\beta8^+$ LNTCs compared with that observed previously (*13*). The A.E7 cells were stimulated for 72 hours and MBP-reactive LNTCs were stimulated for 120 hours and then pulsed for 18 hours with 1 μCi of [^{3}H]thymidine. Incorporation was measured by Betaplate scintography (LKB). Supernatants harvested at 20 hours were assayed for IL-2 bioactivity (*12*). For Trypan blue staining, 5×10^4 A.E7 cells were incubated with 2.5×10^4 DCEK APCs (E^k_α, E^k_β-expressing L cell transfectants) and stained (*11*). For flow cytometric quantitation, cells from six wells were pooled and stained with phycoerythrin (PE)–conjugated mAbs to CD4 and fluorescein isothiocyanate (FITC)–conjugated mAbs to either $V_\alpha11$ for A.E7 cells or to $V_\beta8$ (Pharmingen, San Diego, CA) for MBP-reactive LNTCs, resuspended in 300 μl of flow cytometry buffer [phosphate-buffered saline (PBS) with 1% (w/v) bovine serum albumin, and

0.05% sodium azide], and analyzed for constant time (120 s) on a FACScan cytometer with Lysis II software (Becton Dickinson, Mountain View, CA). For cell cycle analysis, we used 2×10^6 A.E7 cells stimulated for 66 hours by peptide antigen and 2×10^7 splenic APCs. For the last 18 hours BrdU (1 μM) was added. Viable cells were separated by Ficoll gradients, ethanol-fixed, and stained with mAbs to BrdU conjugated to FITC according to manufacturer's instructions (Becton Dickinson). We collected 10,000 events and the data is representative of four experiments.

18. J. Critchfield, M. Racke, M. J. Lenardo, unpublished results. Death was not due to T-T cytotoxicity because it was observed at a high APC to T cell ratio, and mouse T cells lack major histocompatibility complex class II expression. Also, neither the antigen solution itself nor supernatants taken from cultures stimulated with high antigen concentrations were intrinsically toxic.
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20. The MBP-reactive LNTCs were stimulated in vitro with 50 μM Ac1–11 for 48 hours, then purified on lympholyte M (Cedarlane Laboratories, Westbury, NY) gradients. Activated cells were incubated with the indicated amounts of IL-2 for 48 hours. At this time, [^{3}H]thymidine incorporation was measured. The cells were then rinsed and restimulated with 1×10^4 LNTCs with 50-fold excess of irradiated syngeneic (B10.PL) splenocytes per U-bottom well and the indicated concentrations of Ac1–11 peptide. During restimulation IL-2 was included to prevent IL-2–withdrawal apoptosis. Cells were quantitated after 48 hours. Control experiments showed that the Ac1–11 peptide had no intrinsic toxicity on LNTCs. For experiments that used mAbs 3C7 or S4B6, 5×10^4 A.E7 cells were incubated with 5×10^5 splenic APCs, and the indicated amount of peptide antigen and mAb (50 μg per well) was added in a 96-well flat bottom dish. We used A.E7 cells because they enter a resting state and do not undergo withdrawal apoptosis when IL-2 is removed from the culture. Viable cell numbers are the means from duplicate values quantitated by flow cytometry (*10*). The mAb 3C7 [T. R. Malek, G. Ortega, J. P. Jakway, C. Chan, E. M. Shevach, *J. Immunol.* **133**, 1976 (1984)] or S4B6 [T. R. Mosmann, H. Cherwinski, M. W. Bond, M. A. Giedlin, R. L. Coffman, *ibid.* **136**, 2348 (1986)] was purified from ascites by ammonium sulfate precipitation and dialyzed against PBS. The mAb 3C7 was then purified by Sephadex gel filtration chromatography and filtered for sterility. The mAb 11B11 was described previously [J. O'Hara and W. E. Paul, *Nature* **315**, 333 (1985)].
21. The MBP-reactive TCR transgenic splenocytes from unprimed animals were stimulated in vitro with 50 μM Ac1–11 peptide for 96 hours and rinsed, and 3×10^7 cells in 200 μl of PBS were injected into B10.PL or PL/J hosts. Ten days after transfer, cell suspensions from mesenteric and aortic lymph nodes or spleen were stained with FITC-conjugated mAb to V_β8.1,2, PE-conjugated mAb to V_α2 (Pharmingen), and Red 613–conjugated mAb to CD4 (Gibco-BRL), then 5×10^4 $CD4^+$ events were analyzed by FACScan with Lysis II software (Becton Dickinson). Transgenic animals were used from the second or third backcross generation; no evidence of rejection or graft versus host disease was observed. Experiments were done under an approved protocol in accordance with the animal use guidelines of the National Institutes of Health.
22. Nontransgenic animals have been shown to have a limited but heterogeneous group of MBP-reactive TCRs. [J. L. Urban *et al.*, *Cell* **54**, 577 (1988); R. B. Bell, J. W. Lindsey, R. A. Sobel, S. Hodgkinson, L. Steinman, *J. Immunol.* **150**, 4085 (1993)].
23. Donor mice, $(PL \times SJL)F_1$, 8- to 12-week-old females (Jackson Laboratory, Bar Harbor, ME) were primed at the shoulders and flanks with 400 μg of guinea pig MBP in complete Freund's adjuvant as described [M. K. Racke *et al.*, *J. Immunol.* **146**, 3012 (1991)]. Ten days later, draining LNCs were harvested and 8×10^6 cells in 2 ml were stimulated with MBP (25 μg/ml) for 4 days. Cells (3×10^7) in PBS (200 μl) were immediately injected intravenously into syngeneic, naive recipients. Statistical analysis was done by two-sample *t* tests and a Hotelling's T^2 multivariate analysis. Histopathological analysis was done at day 60 after transfer on the dorsal spinal cord at L7 from mice perfused through the heart with 2.5% glutaraldehyde. The histological samples were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Photomicrographs (magnification ×271) were taken from 1-μm sections stained with toluidine blue. Encephalitogenic nontransgenic LNTCs were prepared, then rinsed in RPMI 1640 with 50 mM Hepes (pH 7.5) and stained in 10 ml of DiI (4 μg/ml) (Molecular Probes, Inc., Eugene, OR) for 10 min at 37°C. Cells were then washed three times with RPMI 1640 with 10% fetal calf serum before transfer. Cells were counterstained with FITC-conjugated antibody to CD4 (Pharmingen) and flow cytometry was done (*21*).
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Premature p34^{cdc2} Activation Required for Apoptosis

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Activation of the serine-threonine kinase p34^{cdc2} at an inappropriate time during the cell cycle leads to cell death that resembles apoptosis. Premature activation of p34^{cdc2} was shown to be required for apoptosis induced by a lymphocyte granule protease. The kinase was rapidly activated and tyrosine dephosphorylated at the initiation of apoptosis. DNA fragmentation and nuclear collapse could be prevented by blocking p34^{cdc2} activity with excess peptide substrate, or by inactivating p34^{cdc2} in a temperature-sensitive mutant. Premature p34^{cdc2} activation may be a general mechanism by which cells induced to undergo apoptosis initiate the disruption of the nucleus.

On contact with target cells, cytotoxic T lymphocytes (CTLs) release granule serine proteases that trigger apoptosis (*1–3*). The transmembrane pore-forming protein perforin probably facilitates protease entry into the target cell (*2, 3*). Unlike developmentally regulated programmed cell death, apoptosis induced by cytotoxic granule proteases does not require new protein synthesis (*2, 3*), suggesting that the protease initiates nuclear disintegration through a posttranslational mechanism. The p34^{cdc2} kinase is a highly regulated serine-threonine kinase (*4*) that, when complexed with cyclins A and B, controls cell entry into mitosis; this complex initiates the dissolution of the nuclear membrane and promotes chromatin condensation, events that are also hallmarks of apoptosis (*5, 6*). The resemblance of apoptosis to the "mitotic catastrophe" seen in eukaryotic cells overexpressing p34^{cdc2} at an inappropriate time during the cell cycle (*7, 8*) prompted us to examine the role of this kinase in apoptosis.

We examined the induction of p34^{cdc2} kinase in YAC-1 lymphoma cells by fragmentin-2 in the presence or absence of perforin. Fragmentin-2 is a granule serine protease produced by natural killer (NK) cells that has homology to human cytotoxic T lymphocyte (CTL) granzyme B (*2*). After a 45-min treatment with these agents, p34^{cdc2} was immunoprecipitated from the cell lysates with a polyclonal COOH-terminal specific antibody (*9*) and the kinase activity was measured with two p34^{cdc2} peptide substrates [peptide A, derived from casein kinase II (CKII) (*9*), and peptide B, derived from nucleolin (*10*)] (*11*). In the presence of constant amounts of perforin, fragmentin-2 stimulated kinase activity in a

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