(90 chains each for A-78 and C-78).

The system with unmatched polymer length pairs (A-18/C-78 and A-78/C-18) had a M_w somewhat greater than the simple average of constituent block copolymers (3.9×10^4 g/mol and 4.3×10^4 g/mol for the A-18/C-78 and A-78/C-18 combinations, respectively), indicating an association of ionomeric block copolymers, yet much smaller than that of PIC micelles. Block copolymers in these combinations may assume a minimum number of anion-cation associations to compensate for their charge. If aggregates formed consisting of one longer chain (DP = 78) with four shorter chains (DP = 18), then the M_w of such an association would be $\sim 4 \times 10^4$ g/mol, which is similar to the observed value for the A-18/C-78 and A-78/C-18 systems. As shown in Fig. 2, unmatched pairs form only the minimal charge-neutralized polyion complex, which is unable to grow further into larger PIC micelles, whereas matched length pairs of block copolymers spontaneously assembled into PIC micelles with a considerable association number. Polymer complexes formed between unmatched pairs should be less stable than those formed between matched pairs, and consequently, in the coexisting competitive condition of matched and unmatched pairs, only the matched pairs form bimolecular complexes that grow into PIC micelles. The remaining block copolymers of unmatched length are left in isolated form.

Circular dichroism spectra of resulting PIC micelles indicated that the poly(L-lysine) block, which is optically active (*9*), assumed no particular secondary structure in the micelle. Thus, both poly(α,β-aspartic acid) and poly(L-lysine) segments in the block copolymer behave as polymer strands without any ordered structures during the process of micellization, which indicates that the charge-neutralizing selective recognition observed here is indeed due to the difference in the chain length itself.

The key determinant in this recognition process is the strict phase separation between the PEG corona and the PIC core domain, requiring regular alignment of the molecular junctions between PEG and poly(amino acid) segments at the interface of the two domains. The requirement for charge stoichiometry (neutralization) in the core is another essential factor in this recognition process. This eventually determines the number ratio of participating polyanion and polycation strands in the core and restricts the spatial arrangement of segments in the core. Ion pairs should have an uniform distribution in the PIC core, and unmatched length pairs cannot achieve this without phase mixing of PEG corona and PIC core domains.

The length selection system demonstrated with these flexible ionomer polymer strands provides a new approach for controlling supramolecular assembly. Recently, higher ordered assembly of block copolymers in selective solvents to form spherical, rodlike, and univesicular or lamellar structures was reported (*10*, *11*). Assembly of charged block copolymers in aqueous medium may lead to the formation of similar higher ordered structures through precise recognition based on the chain lengths of charged segments, which may be useful for constructing self-assembled layers based on electrostatic interaction (*12*). Chain length recognition based on PIC formation at two-dimensional interfaces may yield self-assembled layers of block copolymers with definite layer thicknesses modulated by chain lengths in the charged segments of the block copolymer.

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13. The authors thank H. R. Stapert of the University of Tokyo for extensive discussion and D. W. Grainger of Colorado State University for critical reading of the manuscript. Financial support from the Ministry of Education, Science, Sports and Culture, Japan (a Grant-in-Aid for Scientific Research: Priority Area Research Program–Supramolecular Structures) is highly appreciated.

17 August 1998; accepted 20 November 1998

Requirement for Diverse, Low-Abundance Peptides in Positive Selection of T Cells

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Whether a single major histocompatibility complex (MHC)–bound peptide can drive the positive selection of large numbers of T cells has been a controversial issue. A diverse population of self peptides was shown to be essential for the in vivo development of CD4 T cells. Mice in which all but 5 percent of MHC class II molecules were bound by a single peptide had wild-type numbers of CD4 T cells. However, when the diversity within this 5 percent was lost, CD4 T cell development was impaired. Blocking the major peptide–MHC complex in thymus organ culture had no effect on T cell development, indicating that positive selection occurred on the diverse peptides present at low levels. This requirement for peptide diversity indicates that the interaction between self peptides and T cell receptors during positive selection is highly specific.

The immune systems of higher vertebrates generate a diverse population of potential T cell receptors (TCRs) through random rearrangement of gene segments within the TCR loci. The dilemma is in choosing which T cells will contribute to protective immunity without knowing the antigens that they may eventually encounter (*1*). This problem is addressed by evaluating the TCRs of developing thymocytes based on their recognition of the thousands of different self peptides bound to MHC molecules in the thymus. Potentially autoreactive T cells with TCRs that bind with too high an affinity to self peptide–MHC complexes are eliminated by a process called negative selection. The process of positive selection, by which T cells are chosen to mature, results in a population of T cells that interacts with MHC molecules with sufficient, albeit weak, affinity to permit a strong interaction with a particular nonself peptide presented by self MHC. Whether specific interaction with the self peptide is needed to select such a T cell repertoire has been hotly debated. Of particular interest is whether a given self peptide selects a limited number of different TCRs and thus truly shapes the specificity of T cells during positive selection.

A role for particular self peptide–MHC ligands during positive selection of T cells was demonstrated when specific peptides or increasingly complex peptide mixtures were added to

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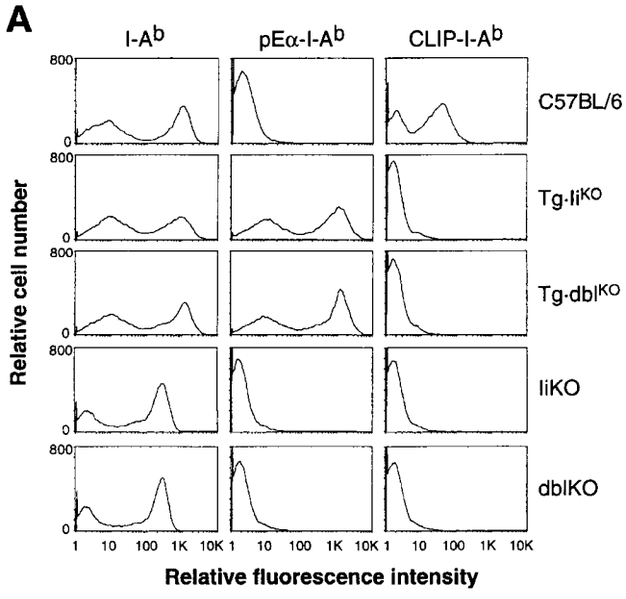
fetal thymic organ cultures (FTOCs), resulting in increasing numbers of CD8 T cells (*2*). In contrast, a number of recent reports analyzing selection of CD4 T cells in vivo have concluded that the recognition of peptide during positive selection is quite degenerate (*3*, *4*). These studies used two strains of mice with a single peptide bound to nearly all of their MHC class II molecules. H-2M knockout mice fail to remove a peptide fragment (CLIP) of the chaperone protein invariant chain (Ii) from class II molecules, resulting in almost exclusive surface expression of this peptide (*5*). A second strain of mice (A^bEp) express an altered MHC class II molecule to which a peptide from the MHC class II I-Eα chain, Eα(52–68) (pEα), has been covalently tethered (*6*). On the basis of the significant number of CD4 T cells that develop in H-2M knockout and A^bEp mice, it has been argued that numerous TCRs can be selected on a single peptide and, therefore, that recognition of peptide during positive selection is degenerate. Although it is clear that the T cell repertoire in these mice is not identical to the wild-type repertoire, it has been reported that the range of specificities selected on the single peptide is very broad (*3*, *4*).

Evidence that non-CLIP peptides are present in H-2M knockout mice (*4*, *7*) and that these peptides may contribute to positive selection (*7*) led us to question whether the T cells in these mice were actually selected on a single peptide. Consequently, we generated transgenic mice to address directly the efficiency of positive selection on a single peptide. We have previously described a system in which specific peptides can be loaded onto MHC class II molecules by insertion into the CLIP region of Ii (*8*). Peptide loading occurs in the endoplasmic reticulum when the Ii-peptide fusion protein binds class II dimers. As the Ii-peptide fusion protein is proteolytically processed, the peptide remains bound in the class II groove and is presented at the cell surface (*8*). We now report the generation of mice expressing a human Ii genomic transgene, in which residues 86 to 102 of Ii have been replaced with pEα (*9*). When expressed in Ii-knockout mice ($Tg{\cdot}Ii^{KO}$), this transgene restored surface expression of the class II allele, $I\text{-}A^b$, to wild-type levels on splenocytes (Fig. 1A). The Eα peptide was efficiently loaded onto $I\text{-}A^b$ molecules, as measured by the pEα-$I\text{-}A^b$–specific monoclonal antibody (mAb), YAe. Immunohistochemical analysis revealed comparable expression of these complexes in the thymus (*10*).

Although H-2M does not promote dissociation of Eα52–68) from $I\text{-}A^b$ (*11*), it is involved in peptide loading, even in the absence of Ii (*12*, *13*). To decrease expression of any potential non-Eα peptides, we bred the Ii-pEα transgene onto the Ii, H-2M double-knockout background ($Tg{\cdot}dbl^{KO}$). Splenocytes from these mice expressed levels of $I\text{-}A^b$ similar to those in $Tg{\cdot}Ii^{KO}$ and wild-type mice and stained brightly with YAe (Fig. 1A). We compared the



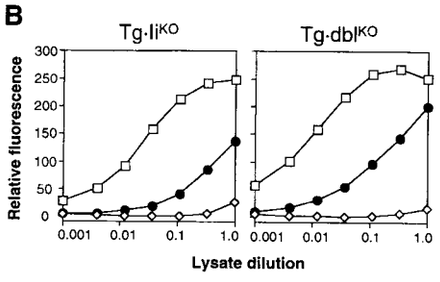


Fig. 1. The Ii-pEα transgene results in high expression of pEα-$I\text{-}A^b$ complexes and restores $I\text{-}A^b$ expression to normal levels in $Tg{\cdot}Ii^{KO}$ and $Tg{\cdot}dbl^{KO}$ mice. (**A**) Expression of $I\text{-}A^b$ complexes in $Tg{\cdot}Ii^{KO}$ and $Tg{\cdot}dbl^{KO}$ mice. Splenocytes from mice with the indicated genotypes were stained with mAbs specific for $I\text{-}A^b$ (Y3P), pEα-$I\text{-}A^b$ complexes (YAe), and CLIP-$I\text{-}A^b$ complexes (15G4) and analyzed by flow cytometry (*23*). (**B**) Quantitation of pEα occupancy in $Tg{\cdot}Ii^{KO}$ and $Tg{\cdot}dbl^{KO}$ mice. $I\text{-}A^b$ protein was measured in $Tg{\cdot}Ii^{KO}$ and $Tg{\cdot}dbl^{KO}$ splenocyte lysates after depletion with Y3P- (◇), YAe- (●), or control immunoglobulin G (IgG)– (□) conjugated Sepharose. Cell lysates were first depleted of Ii-pEα protein-$I\text{-}A^b$ complexes by using the PIN.1 mAb specific for the cytoplasmic tail of human Ii. $I\text{-}A^b$ protein was quantitated by titrating the depleted lysates in an $I\text{-}A^b$ ELISA. The completeness of all depletions was confirmed in corresponding ELISAs. The percentage of class II bound with non-Eα peptides was calculated with DeltaSoft II software by comparing the $I\text{-}A^b$ remaining after depletion with YAe-Sepharose against $I\text{-}A^b$ remaining after depletion with IgG-Sepharose. These percentages ranged from 2 to 9% in three independent experiments and were comparable for both $Tg{\cdot}Ii^{KO}$ and $Tg{\cdot}dbl^{KO}$ splenocyte lysates.

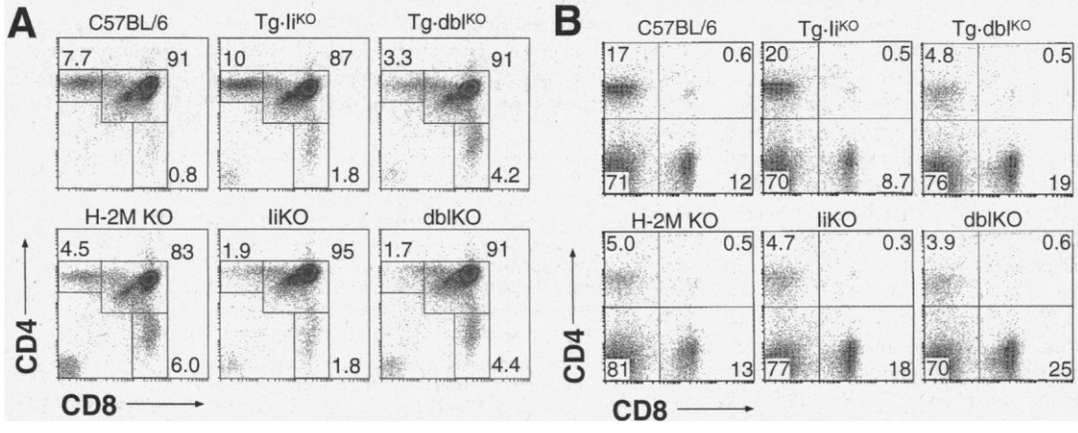


Fig. 2. CD4 T cell development is impaired in $Tg{\cdot}dbl^{KO}$ mice. CD4 versus CD8 plots of (**A**) thymocytes and (**B**) splenocytes from the indicated mice are shown. The percentage of total cells falling within each gate or quadrant is indicated. Cells were stained with mAbs specific for the CD4 and CD8 coreceptors and analyzed by flow cytometry (*24*). The CD4 versus CD8 profiles shown are representative of the six $Tg{\cdot}Ii^{KO}$ mice and eight $Tg{\cdot}dbl^{KO}$ mice analyzed.

pEα occupancy between Tg·Ii^{KO} and Tg·dbl^{KO} mice by depleting cell lysates of pEα-I-A^b complexes and of I-A^b bound to the hybrid Ii protein and quantitating the remaining I-A^b molecules by enzyme-linked immunosorbent assay (ELISA) (Fig. 1B). I-A^b molecules remaining after YAe depletion represent the fraction of class II bound by non-Eα peptides. This approach revealed that Tg·Ii^{KO} and Tg·dbl^{KO} mice had equivalent pEα occupancy, ~95% of total MHC class II molecules.

The self peptide repertoires of both Tg·Ii^{KO} and Tg·dbl^{KO} mice appeared equally skewed toward a single peptide, but the development of CD4 T cells in these two strains of mice was markedly different. Whereas normal numbers of CD4 T cells developed in Tg·Ii^{KO} mice, the percentage of CD4 T cells in Tg·dbl^{KO} mice was reduced by more than 70% in both the thymus [8.2 ± 1.6% in wild type (n = 6), 9.8 ± 2.4% in Tg·Ii^{KO} (n = 6), 2.8 ± 0.4% in Tg·dbl^{KO} (n = 8)] and the spleen [19.8 ± 4.4% in wild type, 23.7 ± 3.9% in Tg·Ii^{KO}, 5.1 ± 1.8% in Tg·dbl^{KO}] (Fig. 2). The specificities of these CD4 T cells were altered from wild type as they proliferated in response to wild-type splenocytes in a mixed lymphocyte reaction (*14, 15*). In addition, two different transgenic TCRs that were selected in wild-type mice failed to be selected in Tg·Ii^{KO} mice, confirming that the CD4 T cell repertoire is altered in Tg·Ii^{KO} mice in spite of the normal number of cells that develop (*14*, *16*).

To explain the difference in CD4 T cell development between Tg·Ii^{KO} and Tg·dbl^{KO} mice, we focused on the small percentage of I-A^b molecules in Tg·Ii^{KO} mice that are not loaded with Eα peptide. To evaluate the peptide component of these I-A^b molecules, we compared the ability of Tg·Ii^{KO} and Tg·dbl^{KO} splenocytes to stimulate T cell hybridomas specific for different endogenous peptide-I-A^b complexes (*13*). As expected, both Tg·Ii^{KO} and Tg·dbl^{KO} splenocytes could stimulate pEα-I-A^b–specific T cells (*10*). However, only Tg·Ii^{KO} splenocytes stimulated each of the other endogenous peptide–specific T cell hybrids (Fig. 3). Thus, in Tg·Ii^{KO} mice, there is a small population of I-A^b molecules loaded with diverse, high-affinity non-Eα peptides that are dependent on H-2M and therefore not detectable in Tg·dbl^{KO} mice. These peptides appear to be critical for positive selection of the normal number of CD4 T cells seen in Tg·Ii^{KO} mice.

This dependence on non-Eα peptides for CD4 T cell development in Tg·Ii^{KO} mice brings into question whether the Eα peptide contributes significantly to the positive selection of CD4 T cells in these mice. To address this issue we cultured Tg·Ii^{KO} fetal thymi in the presence of mAbs that block either all I-A^b complexes (Y3P) or only pEα-I-A^b complexes (YAe). Both of these mAbs prevent T cell interaction with the I-A^b molecules to which they bind (*17*, *18*). As expected, blocking I-A^b molecules with Y3P substantially reduced selection of mature CD4$^+$CD8$^-$TCR^{hi} thymocytes (mean, 53% of control lobes) (Fig. 4). However, blocking only pEα-I-A^b complexes did not reduce the number of CD4 thymocytes. YAe-treated Tg·Ii^{KO} lobes had consistently increased percentages of CD4$^+$CD8$^-$TCR^{hi} thymocytes (mean, 142% of control lobes), perhaps indicative of decreased negative selection due to blocking of the highly abundant pEα-I-A^b complexes. This inability to block selection was not due to poor binding because YAe is a higher affinity mAb than Y3P (*17*). Similarly, in FTOC blocking experiments with Tg·dbl^{KO} mice Y3P significantly blocked CD4 T cell development, whereas YAe had little effect (Y3P, 57% of control; YAe, 95% of control).

These experiments further demonstrate that non-Eα peptides select the majority of CD4 T cells in Tg·Ii^{KO} mice. In addition, the difference between blocking with Y3P versus YAe in Tg·dbl^{KO} FTOCs suggests that non-Eα peptides contribute to selection in Tg·dbl^{KO} mice as well. Our inability to detect non-Eα peptides in Tg·dbl^{KO} mice does not mean that such peptides are absent. Peptides can contribute to both positive and negative selection when present at levels below thresholds for peripheral activation (*19*). Still, we cannot exclude the possibility that some T cells are selected on pEα-I-A^b complexes in Tg·Ii^{KO} and Tg·dbl^{KO} mice. We believe, however, that many of the CD4 T cells in Tg·dbl^{KO} mice are likely selected by small numbers of non-Eα peptides still present in these mice. It is possible that low-abundance peptides are also responsible for a significant percentage of the positive selection seen in H-2M knockout and A^{b}Ep mice.

Our results provide in vivo evidence that a diverse population of MHC-bound self peptides is essential for positive selection of T cells. This conclusion agrees with early in vitro work in the class I system, which described a critical role for peptide diversity in positive selection of CD8 T cells (*2*). Not surprisingly, CD4 and CD8 T cells appear to recognize peptide-MHC complexes in a similar manner during positive selection. The importance of diversity within MHC-bound self peptides for efficient positive selection in vivo demonstrates that the recognition of peptide by a TCR is not promiscuous. In

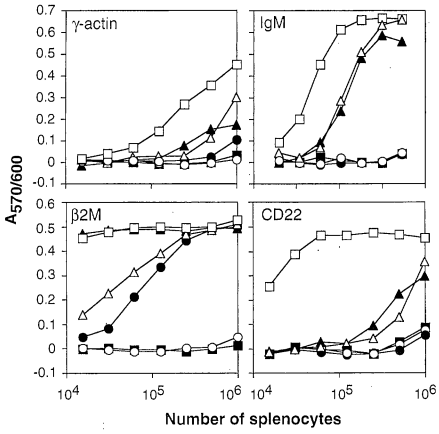


Fig. 3. Non-Eα peptides present in Tg·Ii^{KO} mice are not detectable in Tg·dbl^{KO} mice. The relative expression of different endogenous peptide-I-A^b complexes on splenocytes from C57BL/6 (□), Tg·Ii^{KO} (△), Tg·dbl^{KO} (○), IiKO (▲), dblKO (●), and H-2M KO (■) mice was measured with T cell hybridomas specific for the following peptides: I-Eα(52–68), γ-actin(157–171), IgM(377–392), CD22(25–39), and β_2M(48–58) (*13*). Titrated numbers of splenocytes were cultured with 10^5 T cell hybrids for 18 to 20 hours. Interleukin-2 (IL-2) production was measured with the IL-2–sensitive HT-2 cell line in a colorimetic Alamar Blue assay. Data are presented as the mean absorbance (A) at 570 nm minus A_{600} ($A_{570/600}$) of duplicate cultures.

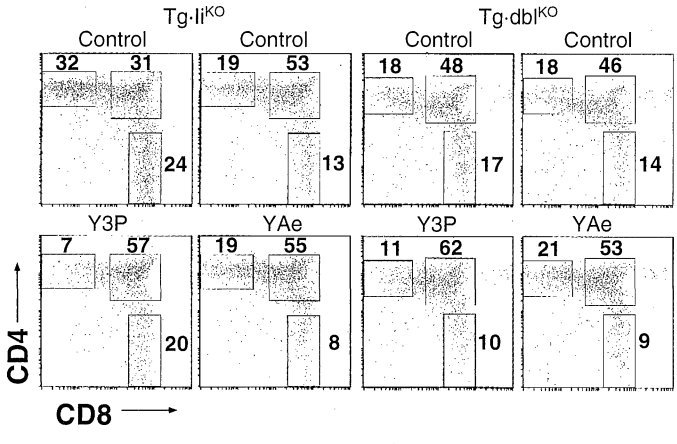


Fig. 4. The Eα peptide does not contribute significantly to positive selection in Tg·Ii^{KO} and Tg·dbl^{KO} mice. CD4 versus CD8 plots of TCR^{hi} gated thymocytes are shown for FTOCs from Tg·Ii^{KO} or Tg·dbl^{KO} mice cultured in the presence of the indicated antibodies. Day 16 fetal thymi were cultured for 7 days in the presence of the different mAbs at a final concentration of 40 μg/ml. Concentrations greater than 40 μg/ml did not increase the efficiency of blocking. One lobe from each thymus was cultured in 15G4 (control) and the other lobe in either Y3P (I-A^b) or YAe (pEα-I-A^b). Media and antibody were replaced daily. On day 7, thymocytes from each culture were stained for CD4, CD8, and TCRβ and analyzed by flow cytometry (*24*). For each thymus, the percentage of blocking was calculated by dividing the percentage of CD4$^+$CD8$^-$TCR^{hi} thymocytes in the treated lobe by the percentage of these cells in the control lobe. Tg·Ii^{KO}: Y3P, 53 ± 11% (n = 10); YAe, 142 ± 25% (n = 9). Tg·dbl^{KO}: Y3P, 57 ± 5% (n = 3); YAe, 95 ± 15% (n = 3).

a normal thymus, a single peptide does not select millions of different TCRs, as has been suggested in the analyses of H-2M knockout and A^bEp mice (*3*, *4*). Instead, the signal through the TCR that eventually leads to positive selection is driven by and dependent on specific interactions with self peptides. This degree of selectivity may be similar to the recognition of peptide during T cell activation. Indeed, it would be reasonable for the immune system to evaluate T cells during development on the basis of the rules of recognition that are required in the periphery.

The specific recognition of peptides appears so central to the generation of a complete T cell repertoire that even peptides present at very low levels can contribute to positive selection of T cells. These peptides generate the bulk of the diversity within MHC-bound peptides and probably support the development of the majority of selected thymocytes. This requirement for diverse, low-abundance peptides suggests that specificity during positive selection is fundamental to the generation of a broad, functional T cell repertoire.

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15. Mixed lymphocyte cultures were performed with C57BL/6 ($H\text{-}2^b$), BALBc ($H\text{-}2^d$), $Tg{\cdot}Ii^{KO}$, and $Tg{\cdot}dbl^{KO}$ splenocytes and lymph node cells. Irradiated (20 Gy) splenocyte stimulator cells (4×10^5 or 2×10^5) were cultured with 4×10^5 lymph node responder cells in each pairwise combination of the four mouse types. After 3 days, the cultures were pulsed with 0.5 μCi of ^{3}H-thymidine for 1 day, harvested, and thymidine incorporation was measured. Both $Tg{\cdot}Ii^{KO}$ and $Tg{\cdot}dbl^{KO}$ lymph node cells developed significant proliferative responses to C57B/6 stimulators.
16. T cell–depleted bone marrow cells (3.5×10^6) from AND (*20*), TCli (*21*), and TEa (*7*) TCR transgenic mice were injected intravenously into irradiated $Tg{\cdot}Ii^{KO}$ mice. These TCRs are specific for PCC(81–104)-I-E^k, human CLIP(85–101)-I-A^b, and Eα(52–68)-I-A^b complexes, respectively. Thymocytes and splenocytes were stained for CD4, CD8, and TCR and analyzed by flow cytometry. Small numbers of CD4 thymocytes, as well as peripheral CD4 T cells, in Tcli→$Tg{\cdot}Ii^{KO}$ and AND→$Tg{\cdot}Ii^{KO}$ chimeric mice indicated that both of these TCRs cannot be selected on $Tg{\cdot}Ii^{KO}$ thymic epithelium. Furthermore, as expected, TCR transgenic T cells in TEa→$Tg{\cdot}Ii^{KO}$ chimeras were deleted.
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23. Erythrocyte-depleted splenocytes were analyzed for expression of MHC class II molecules. Cells were incubated on ice with biotinylated mAbs Y3P (I-A^b, American Type Culture Collection HB183), YAe (pEα-I-A^b) (*17*), and 15G4 [CLIP-I-A^b (*22*)], followed by streptavidin–fluorescein isothiocyanate (FITC) (Vector). Stained cells were analyzed by a FACScan flow cytometer (Becton-Dickinson).
24. Thymocytes or erythrocyte-depleted splenocytes were incubated on ice with anti–CD4-phycoerythrin, anti–CD8α- FITC, and anti–TCRβ-biotin mAbs (all from Pharmingen) followed by streptavidin-Tricolor (Caltag). Stained cells were analyzed by a FACScan flow cytometer (Becton-Dickinson).

25. We thank A. W. Goldrath for helpful discussions and advice, M. J. Bevan and P. J. Fink for comments on the manuscript, S. Eastman for technical assistance, C. Wilson for generation of transgenic mice, and D. Wilson for maintenance of mice. Care of all experimental animals was in accordance with the guidelines of the University of Washington. Supported by a Hall-Ammerer Memorial Fellowship (G.M.B.), the NIH (A.Y.R.), and the Howard Hughes Medical Institute (A.Y.R.).

7 October 1998; accepted 30 November 1998

Blockade of NMDA Receptors and Apoptotic Neurodegeneration in the Developing Brain

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Programmed cell death (apoptosis) occurs during normal development of the central nervous system. However, the mechanisms that determine which neurons will succumb to apoptosis are poorly understood. Blockade of *N*-methyl-D-aspartate (NMDA) glutamate receptors for only a few hours during late fetal or early neonatal life triggered widespread apoptotic neurodegeneration in the developing rat brain, suggesting that the excitatory neurotransmitter glutamate, acting at NMDA receptors, controls neuronal survival. These findings may have relevance to human neurodevelopmental disorders involving prenatal (drug-abusing mothers) or postnatal (pediatric anesthesia) exposure to drugs that block NMDA receptors.

Glutamate promotes certain aspects of neuronal development, including migration, differentiation, and plasticity (*1*). In the first 2 weeks of neonatal life in the rat, the NMDA subtype of glutamate receptor undergoes a period of hypersensitivity, in which neurons bearing NMDA receptors are rendered highly sensitive to excitotoxic degeneration (*2*). During this period, NMDA receptors are primary mediators of glutamatergic fast excitatory neurotransmission in the brain (*3*). Although NMDA receptor activation can promote survival of cerebellar granule cells in vitro (*4*) or dentate granule neurons in vivo (*5*), evidence that even transient inactivation of NMDA receptors can be lethal for many neurons has not been described. We now report that during a specific stage in ontogenesis coinciding with the period of NMDA receptor hypersensitivity, transient

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