addition of 50 μl of protein G–Sepharose beads followed by incubation for 4 hours at 4°C. Immunoprecipitates were then washed five times with lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled for 5 min. The samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (10% gel) and transferred to a nitrocellulose membrane. Analysis was performed by repeated stripping of the membrane and successive probing with anti-NR1 (5 μg), anti-Src (1:500 dilution), or anti-Kv3.1 (1:4000 dilution). Sources of antibodies: anti-NR1 (mouse monoclonal anti-NMDAR1): Pharmingen; anti-Src (monoclonal antibody 327): J. Bolen, DNAX, Palo Alto, CA; nonspecific mouse IgG, Calbiochem, San Diego, CA; and anti-Kv3.1 (rabbit antisera): T. Perney, Rutgers University.

15. M. Sheng, J. Cummings, L. A. Roldan, Y. N. Jan, L. Y. Jan, *Nature* **368**, 144 (1994).
16. T. M. Perney and L. K. Kaczmarek, *Semin. Neurosci.* **5**, 135 (1993).
17. Whole-cell recordings were made from cultured dorsal horn neurons as described [Y. T. Wang and M. W. Salter, *Nature* **369**, 233 (1994)]. The cultures were bathed in an extracellular solution composed of 140 mM NaCl, 5.4 mM KCl, 33 mM glucose, 1.3 mM $CaCl_2$, 10 mM Hepes, 0.001 mM TTX, 0.01 mM bicuculline, and 0.01 mM strychnine (pH 7.25, 310 to 320 mosM). The recording pipette (4- to 7-megohm resistance) contained intracellular solution composed of 90 mM Cs_2SO_4, 35 mM CsOH, 1.3 mM $CaCl_2$, 11 mM EGTA, 10 mM Hepes, and 4 mM MgATP (pH 7.25, 285 to 305 mosM). EPQ(pY)-EEIPIA, EPQY-EEIPIA, anti-src1, Src(40–58), or sSrc(40–58) were added to the intracellular solution as required. Whole-cell currents were recorded with an AxoPatch 1D amplifier (Axon Instruments) that were digitized (33 KHz) and stored on videotape. Detection and analysis of mEPSCs was done off-line (Synaptic Toolbench) after alignment of their rising edges. Mean mEPSCs contained at least 30 traces. Traces with more than one mEPSC per 200-ms recording period, typically 1 to 5% of the total, were not included in the analysis. Under the present recording conditions, spontaneously occurring mEPSCs were observed in most cells. In all cells, the frequency of the mEPSCs was facilitated by the local application of hypertonic sucrose solution. The slower component of the mEPSCs was blocked by D,L-2-amino-5-phosphonovaleric acid and the fast component was abolished by 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 10 μM).
18. J. M. Bekkers and C. F. Stevens, *Nature* **341**, 230 (1989).
19. S. G. N. Grant *et al.*, *Science* **258**, 1903 (1992); P. Soriano, C. Montgomery, R. Geske, A. Bradley, *Cell* **64**, 693 (1991).
20. I. S. Moon, M. L. Apperson, M. B. Kennedy, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3954 (1994); L. F. Lau and R. L. Huganir, *J. Biol. Chem.* **270**, 20036 (1995).
21. H.-C. Kornau, L. T. Schenker, M. B. Kennedy, P. H. Seeburg, *Science* **269**, 1737 (1995); M. D. Ehlers, S. Zhang, J. P. Bernhardt, R. L. Huganir, *Cell* **84**, 745 (1996).
22. B. Edmonds and D. Colquhoun, *Annu. Rev. Physiol.* **5**, 495 (1995).
23. D. Lieberman and I. Mody, *Nature* **369**, 235 (1994) .
24. A. R. Boxall, B. Lancaster, J. Garthwaite, *Neuron* **16**, 805 (1996).
25. G. Superti-Furga and S. A. Courtneidge, *Bioessays* **17**, 321 (1995).

26. We thank S. Courtneidge, J. Bolen, T. Perney, and R. Wenthold for antibodies; J. F. MacDonald, T. Pawson, and J. C. Roder for critical comments on the manuscript; and Y. De Koninck for software used in analysis. Supported by a grant from the Medical Research Council (MRC) of Canada (M.W.S.) and by the Nicole Fealdman Memorial Fund. M.W.S. is an MRC Scholar, X.-M.Y. is supported by a Fellowship from the Spinal Cord Research Foundation of the Paralyzed Veterans of America, and R.A. is a Fellow of the Rick Hansen Man in Motion Foundation. We thank J. L. Hicks for preparing and maintaining cell cultures and for technical assistance.

2 August 1996; accepted 22 November 1996

Positive Selection of T Cells Induced by Viral Delivery of Neopeptides to the Thymus

Naoko Nakano, Ronald Rooke, Christophe Benoist, Diane Mathis

The relation between an antigenic peptide that can stimulate a mature T cell and the natural peptide that promoted selection of this cell in the thymus is still unknown. An experimental system was devised to address this issue in vivo—mice expressing neopeptides in thymic stromal cells after adenovirus-mediated delivery of invariant chain–peptide fusion proteins. In this system, selection of T cells capable of responding to a given antigenic peptide could be promoted by the peptide itself, by closely related analogs lacking agonist and antagonist activity, or by ostensibly unrelated peptides. However, the precise repertoire of T cells selected was dictated by the particular neopeptide expressed.

Mature T lymphocytes display a biased repertoire of T cell receptors (TCRs), enriched for those that can recognize foreign antigens presented by the animal's own major histocompatibility complex (MHC) molecules and largely purged of those capable of seeing self-antigens in the context of self-MHC molecules (*1*). These biases reflect positive and negative selection processes that take place in the thymus, both controlled by interactions between the TCRs on differentiating thymocytes and the MHC molecules on stromal cells. Positive selection is of particular interest because it shapes the T cell repertoire to deal with antigens not encountered until some time in the future, or even never. Other than that MHC molecules are involved, the precise nature of the ligands that promote positive selection remains undefined.

One system used to address this issue relies on the addition of peptides to fetal thymic organ cultures (FTOCs) derived from mouse strains carrying mutations that interfere with peptide loading and surface display of MHC class I molecules (*2–10*). These studies agreed that peptides are required for positive selection, but disagreed on the nature of the most effective peptides and on their precise role. In addition, this approach has limitations: First, the FTOC systems can give an indication of which peptides are capable of enhancing the selection of a given TCR, but do not address those actually responsible for selection; second, as discussed (*11*, *12*), such systems create a situation in which thymocytes are exposed to few MHC molecules heavily loaded with a single peptide, unlike the natural condition where many MHC molecules carry a heterogeneous mix of peptides.

An alternative approach allows the identification of T cells selected in vivo on a defined peptide. Mouse strains were engineered that express MHC class II molecules loaded with a single peptide—either by introduction of a transgene that encodes a class II chain covalently linked to a particular peptide (*13*) or by generation of a null mutation of the H-2M gene, resulting in class II molecules filled with a peptide derived from the invariant chain (Ii) (*14*, *15*). Surprisingly, results from these studies were largely in agreement: A single peptide promoted selection of a large number of T cells (*13–15*) with a diverse repertoire of TCRs (*13*, *14*). This approach has overcome one of the criticisms of the experiments that use FTOCs but remains susceptible to the second in that these systems produce a situation in which low (*13*) or normal (*14*, *15*) amounts of MHC molecules are loaded with essentially one peptide.

To surmount both limitations, we injected mice intrathymically (i.t.) with an adenovirus vector that directs expression of a peptide of choice (neopeptide) in a form that favors its presentation by MHC molecules on thymic stromal cells. The vector is a nonreplicating derivative of the Ad5 strain, bearing a deletion of the critical E1a and b region (Fig. 1A), which was replaced by a cassette that controls expression of a given cDNA in cells that normally express MHC class II molecules (Fig. 1B). Intrathymic injection of adenoviruses carrying cDNAs within this cassette (*16*) results in measurable protein expression 10 days later, remaining detectable for about 4 weeks. Proteins are expressed in amounts similar to those of endogenously encoded MHC class II molecules and occur primarily in the class II–positive epithelial cells of the cortex and medulla. Expression is confined to an area about 3 to 20% of the injected lobe, does not "spread" to an adjacent uninjected lobe, and is not detectable in the peripheral lymphoid organs. Thus, this system permits one to influence thymic selection quite specifi-

Institut de Génétique et de Biologie Moléculaire et Cellulaire (INSERM, CNRS, Université Louis Pasteur), 1 rue Laurent Fries, 67404 Illkirch, C.U. de Strasbourg, France.

cally, in the absence of effects on peripheral T cell expansion or antigen stimulation.

We cloned into the expression cassette cDNAs encoding Ii or a set of fusion proteins composed of Ii, a cathepsin cleavage site, and one of several peptides that bind to E^k, the last two components joined in series near the COOH-terminus of Ii (Fig. 1, B and C). Invariant chain was used as a peptide "escort" because it facilitates the egress of MHC class II molecules from the endoplasmic reticulum and Golgi apparatus and targets them to endosomal-lysosomal compartments, where they are loaded with peptides (*17*). Hence, it seemed likely that fusion of a neopeptide to Ii would facilitate its delivery to subcellular compartments where it could be loaded efficiently onto MHC class II molecules. Spleen cells from B10.BR mice bearing a homozygous null mutation of Ii ($Ii^{-/-}$) (*18*) were infected with various amounts of a virus expressing a fusion of Ii and amino acids 88 to 103 of moth cytochrome c (MCC). The ability of spleen cells to stimulate 2B4, an MCC-specific T cell hybridoma, was compared with that of control virus-infected splenocytes to which various amounts of the MCC peptide were added exogenously. The Ii-MCC virus-infected spleen cells presented the peptide originating from the fusion protein as efficiently as control virus-infected cells exposed to exogenous peptide concentrations of 0.1 to 0.3 μM. Thus, Ii is an effective "escort" of neopeptides joined to its COOH-terminus, consistent with previous experiments (*19*, *20*).

We used this system to study the positive selection of T cells capable of responding to the MCC(88–103) peptide in the context of E^k. The advantages of working with the anti-MCC response are that it is well characterized (*21*), its evaluation is facilitated by many existing reagents (*22*), it exhibits a highly restricted TCR V_α and V_β usage (*23*), and its selection is totally dependent on Ii expression by thymic stromal cells (*24*). Hence, $Ii^{-/-}k$ mice should be appropriate low-background virus recipients: Their antigen-presenting cells (APCs) express one-third as many E^k complexes at the cell surface as do those from wild-type animals, they have 40% wild-type numbers of mature $CD4^+8^-$ thymocytes, but they lack T cells capable of responding to the MCC peptide in proliferation assays (*24*).

We first determined whether the antigenic peptide or analogs could select a response. $Ii^{-/-}k$ mice were injected i.t. with a control virus (dl324), with one expressing Ii alone (Ii), or with viruses expressing Ii fused to the MCC sequence (Ii-MCC) or to an analog with a substitution at one of the sites expected to contact the TCR (Ii-99E, Ii-102E) (*25*). Three weeks after infection animals were primed with the MCC peptide, and 10 days later draining lymph node cells were challenged in vitro with the same peptide (*26*). Data from typical experiments (Fig. 2, A and B) and results from a large series of studies (Fig. 2C) are shown. Despite significant variation, attributable to the high variability of infection after i.t. injection of virus (*16*), several effects were observed. Injection of the Ii virus resulted in responses, usually weak, in some animals. Delivery of Ii alone might have been expected to complement the defect in $Ii^{-/-}k$ mice and always promote a strong anti-MCC response. However, this did not occur in most animals because expression of the Ii cDNA was controlled by an MHC class II promoter, which is much weaker than that of the endogenous Ii gene. In contrast, introduction of the Ii-MCC virus resulted in responses, often quite robust, in most animals. Injection of either the Ii-99E or Ii-102E viruses also frequently resulted in responses. Thus, a response to the MCC(88–103) peptide can be promoted by i.t. delivery of the antigenic peptide or by analogs thereof.

We next investigated the degeneracy of this phenomenon—whether peptides with very different sequences can promote an anti-MCC response or whether MCC peptides can promote responses to divergent peptides. In one set of studies (Fig. 2C), mice were injected i.t. with viruses expressing fusions of Ii to either residues 67 to 76 of hemoglobin (Ii-Hb) or residues 12 to 26 of λ repressor (Ii-λ). The response to the latter in B10.BR mice is enriched for T cells expressing $V_\beta3$, similar to the response to MCC(88–103) (*27*). Infection with the Ii-Hb virus resulted in a response to MCC immunization in most animals, whereas introduction of the Ii-λ virus had no effect. In a second set of experiments, mice injected i.t. with either the Ii-MCC or Ii-99E virus were challenged with either the λ peptide or the bovine ribonuclease (RNase) (residues 90 to 105) peptide (Fig. 2C). The λ and RNase peptides were chosen because, of the many E^k-binding peptides we screened,

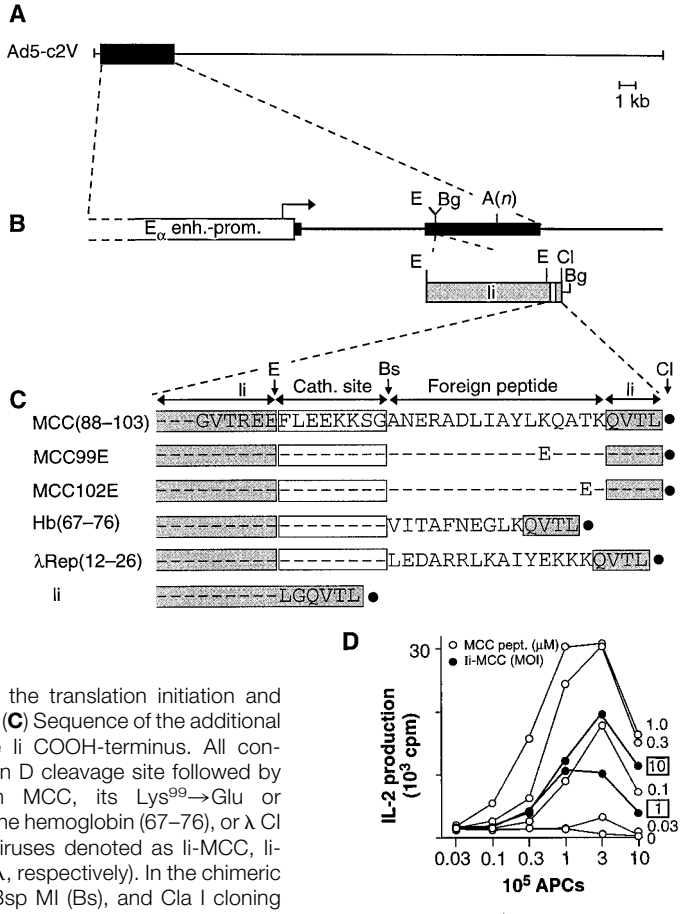


Fig. 1. Adenovirus-mediated delivery of Ii-peptide fusions. (**A**) The recombinant adenovirus Ad5-c2v derives from the dl324 mutant of Ad5 (ΔE1, ΔE3), by replacement of the E1 genes by a cassette that directs expression in MHC class II–positive cells (*33*). (**B**) This cassette is composed of the enhancer-promoter from the E_α gene, an intron and splice signals from the β-globin gene, the cDNA cloning site, and the polyadenylation site from the β-globin gene. The recombinant Ii cDNA fragment cloned between the Eco RI and Bgl II (E, Bg) sites of the cassette contains the entire p31 Ii cDNA, with artificial Eco RI and Cla I (Cl) sites immediately outside the translation initiation and stop codons, respectively. (**C**) Sequence of the additional peptides added onto the Ii COOH-terminus. All constructs contain a cathepsin D cleavage site followed by sequences derived from MCC, its Lys^{99}→Glu or Thr^{102}→Glu variants, murine hemoglobin (67–76), or λ CI (12–26) (in recombinant viruses denoted as Ii-MCC, Ii-99E, Ii-102E, Ii-Hb, and Ii-λ, respectively). In the chimeric cDNAs, artificial Eco RI, Bsp MI (Bs), and Cla I cloning sites were introduced for easy sequence exchange. (**D**) Splenocytes from $Ii^{-/-}k$ mice (*18*) were infected with Ii-MCC virus at multiplicities of infection (MOIs) of 1 and 10 (filled circles) or with dl324 at an MOI of 10 (open circles). After a 72-hour culture, the cells were tested for their ability to stimulate IL-2 production by the MCC-reactive hybridoma 2B4 (*32*), after addition of various doses of MCC peptide for dl324-infected cells. [Similar experiments with the same peptide sequence substituted for the CLIP segment of Ii did not result in significantly enhanced presentation (*20*).] Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

these two elicited responses that depended most critically on expression of Ii by APCs (*20*). Infection with either the Ii-MCC or the Ii-99E virus resulted in a response to the RNase peptide, but not to the λ peptide, in most animals. Thus, the delivery of neopeptides to thymic stromal cells has broad consequences on the T cell repertoire, but some specificity is still evident.

The appearance of new responses after expression of neopeptides in the thymus suggested that the peptides promoted positive selection of new T cells. However, it was necessary to rule out the possibility that display of the neopeptides on thymic stromal cells merely provoked the expansion of preexisting cross-reactive cells. We first examined this at the population level. Mice were injected i.t. with the Ii-99E or the Ii-102E virus; they were then primed with the MCC peptide, and their lymph node cells were challenged in vitro with MCC or the appropriate analog. After infection with either virus, an in vitro secondary response was made to the MCC peptide but not to the analog expressed in the thymus (Fig. 3A). (This result is not comparable with that in Fig. 2, A and C, which shows an anti-MCC response after i.t. Ii-MCC injection and MCC priming, because in those experiments the same peptide selected, primed, and challenged the T cells.) We also addressed this concern at the clonal level, with MCC-reactive T cell hybridomas obtained from mice that had been injected i.t. with either the Ii-99E or the Ii-Hb virus (below). In neither case were the hybridomas able to react to the peptide that had been expressed in the thymus (Fig. 3B). Thus, the appearance of new T cell responses cannot be explained by virus-delivered neopeptides merely expanding a population of preexisting cross-reactive cells. It was also important, especially in the case of the anti-MCC response of animals infected with the Ii-MCC virus, to establish that the same epitope was being generated from the endogenously expressed Ii-peptide fusion protein and from the peptide added exogenously. T cell hybridomas derived from Ii-MCC–infected animals could respond to both virus-infected and peptide-exposed splenocytes (Fig. 3C) and were also stimulated by splenic and thymic APCs from transgenic mice carrying the Ii-MCC cDNA inserted in the same expression cassette (*20*).

The results from these control studies were consistent with the intepretation that the novel responses reflected the positive selection of new T cells. However, when evaluated by means of the lymph node proliferation assay, the responses were variable and sometimes weak. To verify the selection of new cells, we derived sets of MCC-reactive T cell hybridomas in parallel from unmanipulated $Ii^{+/-}$ heterozygotes (phenotypically wild-type) or from mice injected i.t. with different viruses. Only one hybridoma could be obtained from the mice injected with the Ii virus. Sets of hybridomas from the other ani-

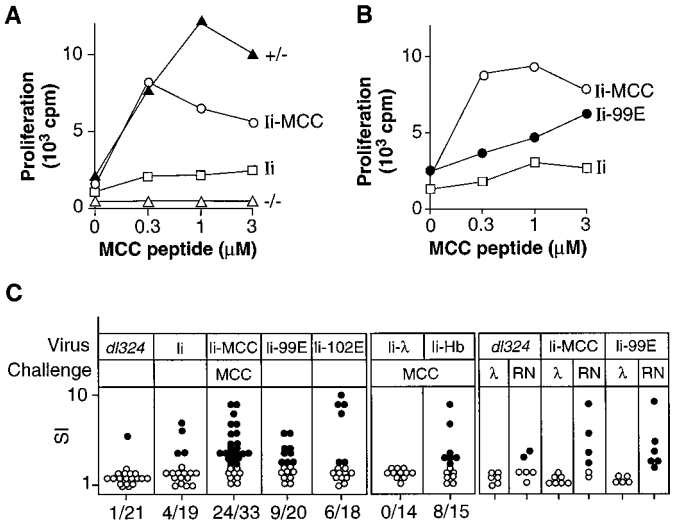


Fig. 2. New T cell responses after i.t. delivery of neopeptides. (**A** and **B**) In two independent experiments, $Ii^{-/-}k$ homozygous mutant mice (*18*) were infected i.t. with the indicated viruses, and lymph node responses to MCC immunization were tested 25 days later (*26*); −/−, uninfected $Ii^{-/-}k$ mouse; +/−, $Ii^{-/-}k$ heterozygote littermate (phenotypically wild-type). (**C**) A compilation of many experiments similar to those of (A) and (B). (Left and middle) $Ii^{s}k$ mice were infected with the virus shown, then primed with MCC peptide, and lymph node cells were restimulated in vitro with MCC peptide (*26*). The maximum stimulation indices (SIs) from individual mice are shown as dots, with the proportion of responding mice (solid dots) over the course of many experiments indicated. An SI of ≥2.5 was considered a positive response. We eliminated from consideration the few data points that gave artificially high SIs because of total incorporation below 2500 cpm. (Right) $Ii^{-/-}k$ mice after infection were primed in one footpad with MCC peptide, and in the other with λ or RNase peptides; draining lymph node cells were then restimulated in vitro with the same peptide. We thus considered data only from mice shown to be successfully reconstituted by the presence of a significant response to MCC peptide. Control +/− mice immunized with the same peptides gave an SI of 4.5 to 17 (mean, 9.1) (MCC peptide), 2.4 to 6.0 (mean, 4.1) (λ peptide), or 3.1 to 13 (mean, 8.3) (RNase peptide).

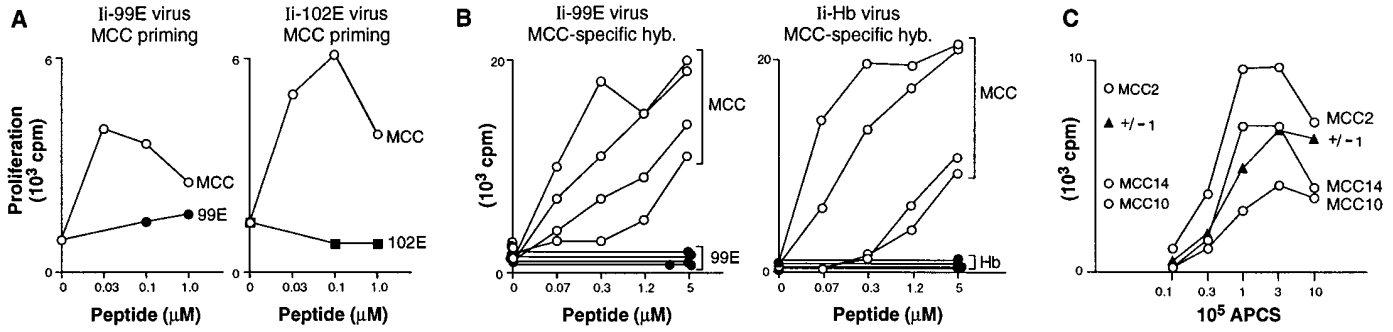


Fig. 3. Evidence against expansion of cross-reactive cells and reactivity to an alternative epitope. (**A**) $Ii^{-/-}k$ mice were infected i.t. with Ii-99E or Ii-102E viruses and then were primed subcutaneously with MCC peptide after 25 days. Ten days later, popliteal lymph node cells were stimulated in vitro with MCC peptide or its 99E or 102E analogs, as indicated (*26*). (**B**) T hybridoma cells obtained from $Ii^{-/-}$ mice infected i.t. with Ii-99E or Ii-Hb virus (*34*) were challenged with MCC, 99E, or Hb peptides, as indicated, and activation displayed as IL-2 secretion (*32*). (**C**) $Ii^{-/-}k$ splenocytes were infected with Ii-MCC virus and used, after 72 hours of culture, as APCs to stimulate different T hybridomas, originating from mice infected i.t. with Ii-MCC (MCC2, 10, 14) or from a control mouse (+/− 1; for T hybridoma identity, see Fig. 4). Positive control stimulation by control virus-infected splenocytes supplemented with 1.0 μM MCC peptide is represented by isolated dots.

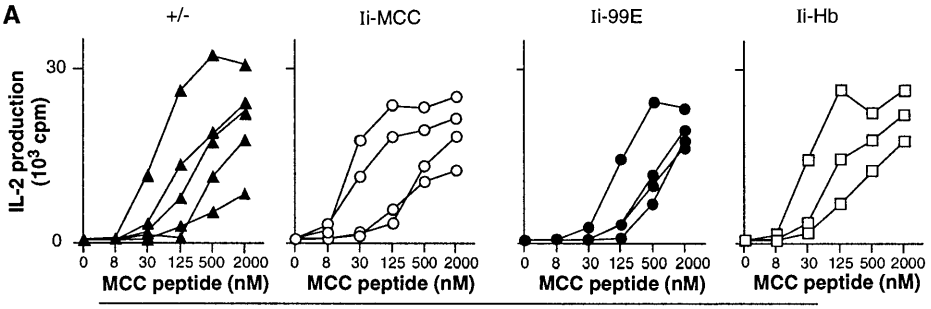
mals were compared for several diagnostic features.

Dose-response curves for the different hybridoma sets (Fig. 4A) are largely overlapping, suggesting that the T cells selected on an unknown natural peptide and those elicited after i.t. expression of various neopeptides have receptors of similar affinities. However, the hybridomas from Ii-99E–injected mice did appear to be biased toward lower reactivities.

We also examined fine specificities, assaying either agonist or antagonist activity (Fig. 4B). The different hybridoma sets showed distinct patterns of cross-reactivity, especially when those from unmanipulated mice were compared with those from animals expressing neopeptides. There were multiple examples of cross-reactivity with hybridomas from control (+/−) mice, consistent with published reports (*28*). In contrast, the hybridomas from animals injected with the Ii-MCC virus rarely showed cross-reactivity, whereas those from animals injected with the Ii-99E virus frequently responded to MCC variants at position 102. Hybridomas from animals injected with the Ii-Hb virus were rarely cross-reactive. In the assays of antagonist activity (Fig. 4C), the 99E peptide did not inhibit the anti-MCC response of T cells (either hybridomas or a cloned line) from mice injected with the Ii-99E virus, whereas the 99R peptide was antagonistic in two instances. Likewise, the Hb peptide did not antagonize the response to MCC by cells from mice infected with the Ii-Hb virus (*20*). Thus, a peptide that promotes the development of a T cell need not be an antagonist of it.

Lastly, TCR usage was explored (Figs. 4B and 5). The response of B10.BR mice to pigeon cytochrome c is highly restricted: Most responding cells express $V_\alpha 11$ and $V_\beta 3$; the V_α complementarity-determining region (CDR)–3 is usually eight residues long with a conserved glutamic acid at position 93 and serine (or related amino acid) at position 95; the V_β CDR-3 is usually nine residues long with a conserved asparagine at position 100 and alanine or glycine at position 102 (*21*, *23*). The MCC-reactive hybridomas from our control mice displayed these features, as did the single hybridoma from mice injected with virus expressing Ii alone (*20*). The hybridomas from animals injected i.t. with the Ii-MCC virus appeared to be a mix of cells displaying these "canonical" TCRs (three of seven sequences) and those with a more diverse repertoire of receptors (four of seven sequences)—including different V_αs and V_βs, greater variation in CDR-3 lengths, and alternate amino acids at conserved positions. The TCRs on hybridomas from animals injected with the Ii-99E virus differed from the canonical TCRs to an even greater extent. Not a single TCR had both $V_\alpha 11$ and $V_\beta 3$; when either one or the other conserved V element was present, it almost always had a CDR-3 of nonstandard length or amino acid composition or both. Instead, half of the receptors had $V_\beta 8$, which was paired with either $V_\alpha 11$ or $V_\alpha 8$. The hybridomas from mice injected with the Ii-Hb virus were again different: V_α usage was enriched in $V_\alpha 11$s with canonical CDR-3s; V_β usage was variable although there were two canonical CDR-3s (Hb7 and HB50). Taken together, the data on fine specificity and TCR usage imply that each virus has promoted the positive selection of a different repertoire of MCC-reactive T cells, one that also diverges from that of unmanipulated B10.BR mice.

Thus, in this system, the peptide that stimulates a given T cell can also promote its selection, as i.t. injection of a virus expressing the Ii-MCC fusion protein promoted a



B

Selected with	Hybridoma	Cross-reactive to: 99A	99R	99Q	99E	102E	102L	Antagonists	V_α	V_β
None (wild type)	+/-1							—	11	3
	+/-8			+	+			—	11	3
	+/-15							—	11	3
	+/-29.3			+	+	+		—	11	14
	+/-29.5						+	NT	11	3
Ii-MCC	MCC2							—	11	3
	MCC6							—	?	6
	MCC8							99R	?	3
	MCC9						+	—	11	3
	MCC11							—	11	16
	MCC14						+	—	10	9
	MCC25							(99R, 99E)^W	11	3
Ii-99E	99E18/5					+	+	—	8	8
	99E29.1							99R	11	1
	99E29.2					+	+	—	8	8
	99E4C						+	99R	1	3
	99EC1	+	+				+	—	11	8
	99EC13							—	11	16
Ii-Hb	Hb2							—	?	8
	Hb7							—	11	3
	Hb50							NT	11	14
	Hb60							NT	11	16
	Hb70						+	NT	?	1
	Hb80							NT	11	14

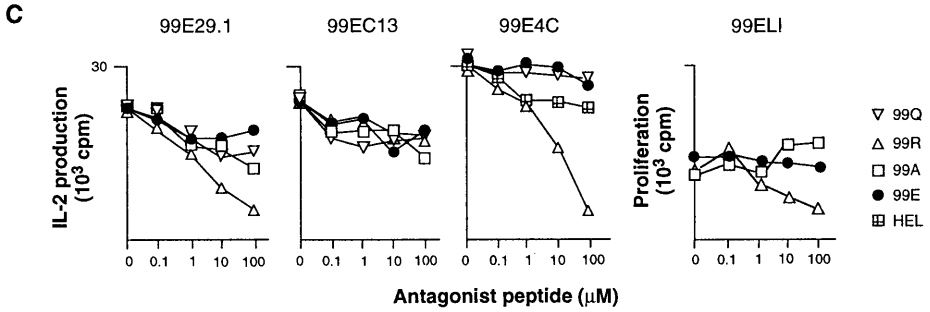


Fig. 4. Properties of hybridomas derived from mice expressing different neopeptides. Sets of MCC-reactive T hybridomas were isolated over the course of five independent fusions, originating from wild-type heterozygotes or from $Ii^{+/-}k$ mice infected i.t. with Ii-MCC, Ii-99E, or Ii-Hb, as shown (*34*). (**A**) The hybridomas were exposed to a range of MCC peptide concentrations. (**B**) Each hybridoma was tested for cross-reactivity to a panel of single-substitution analogs of MCC peptide (at 3.0 μM); for antagonist activity (*35*) (w, weak antagonism for 99R and 99E with MCC25; NT, not tested); and for V_α and V_β usage determined by staining with specific monoclonal antibodies or, in some instances, by direct TCRβ sequencing [?, not determined (not $V_\alpha 11$, 8, or 3)]. (**C**) Typical antagonist assays (*35*) for four cloned T cells derived from Ii-99E–injected mice [three hybridomas identified above and a T cell clone (99EL1) maintained by serial restimulation].

healthy MCC response. The T cells responding to APCs fed MCC exogenously also reacted to splenic or thymic APCs expressing the Ii-MCC fusion, arguing against the idea that the precise peptide sequences involved in positive selection and peripheral stimulation were somehow different. By several criteria, the repertoire of MCC-reactive T cells selected on the Ii-MCC fusion was distinct from that of B10.BR mice selected on an unknown natural peptide. One could argue that the differences reflect negative selection of the higher affinity TCRs in mice expressing the Ii-MCC fusions in thymic stromal cells; indeed, injection of this virus into mice carrying the rearranged TCR genes from the 2B4 hybridoma provokes clonal deletion of this specificity (*20*). However, negative selection of high-affinity receptors may not be an adequate explanation for the differences. The hybridoma dose-response curves showed that the cells selected in +/− control and Ii-MCC–injected animals had overlapping sensitivities to MCC. In addition, the V_β usage of the $V_\beta3$-negative MCC-selected hybridomas differed markedly from that of B10.BR mice injected from birth with a bacterial superantigen that deletes $V_\beta3$: heterogeneous in the former, almost solely $V_\beta8$ in the latter (*27*). Regardless of whether the distinct repertoires of MCC-reactive cells in the two types of mice reflect negative selection effects or the influence of different positively selecting peptides, our data demonstrate that the same peptide that stimulates a peripheral T cell can promote its positive selection in the thymus. This provides important in vivo confirmation of results from certain of the FTOC systems (*3*, *8*–*10*) and highlights the issue of how the same TCR–MHC-peptide recognition event can produce different outcomes (positive or negative selection, or activation). The finding that peptides capable of promoting positive selection can range from neutral to fully stimulatory to mature T cells is incompatible with a simple affinity model.

Closely related analogs of an antigenic peptide can also promote selection of antigen-responsive T cells. These cells differ (in fine specificity, TCR usage, and perhaps average affinity) from those selected both by the antigenic peptide itself and by the unknown natural peptides that normally select the response. Antagonist activity and selecting ability do not correlate in this in vivo system, as they were reported to do in one of the MHC class I–restricted FTOC systems (*5*–*7*). It is not yet clear whether this reflects a difference between antigens, MHC restriction elements, or merely experimental systems.

Positive selection in this system is promiscuous, consistent with a report that selection of T cells expressing a particular TCR is promoted by multiple structurally divergent peptides in an FTOC system (*10*); it is also reminiscent of the results on mice displaying MHC molecules filled with essentially a single peptide, where a large—but not complete—T cell repertoire was selected, on the basis of size, response capacity, and inclusion of specific TCR specificities (*13*, *14*, *29*). However, these results are at odds with the high degree of specificity reported with other FTOC systems (*3*, *5*). In considering these discrepancies, it may be instructive to draw an analogy with the range in degeneracy of the responses made by mature T cells: Reactivity to a single peptide may be restricted to a very limited subset of TCRs or may involve a large diversity of receptors (*30*); a T cell displaying a particular TCR may react fastidiously to a single peptide or promiscuously to multiple peptides with very different sequences (*31*). Positive selection of immature T cells might exhibit a similar range in degeneracy, perhaps explaining the divergent results with different systems. In this context, it is interesting to consider the highly restricted anti-MCC response: Does the predominance of $V_\alpha11^+V_\beta3^+$ TCRs with canonical CDR-3s reflect biases imposed by repertoire selection in the thymus or requirements of MCC(88–103) recognition by mature T cells? Our results suggest that antigen recognition may play some part (since there is an overall enrichment for $V_\alpha11^+$ TCRs in our hybridomas) but that repertoire selection must be the driving force. We hypothesize that the canonical $V_\alpha11^+V_\beta3^+$ receptors reflect selection by a dominant MHC-peptide complex, absent in Ii-deficient mice and not mimicked by the neopeptides assayed so far. Such an explanation would be consistent with results from bone marrow chimera experiments (*28*).

Thus, virus-mediated expression of a

Hybridoma	V_α	CDR3	J_α	V_β	CDR3	J_β	
+/- 29-3	$V_\alpha11$	C A A TGTGCTGCT **E A S** G S W Q L GAGGCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA 22	$V_\beta14$	C A W TGTGCCTGG **S L N** W **G** Q D T Q AGTCTAAALTGGGGGCAAGACACCCAG	Y F G TACTTTGGG	2.5
+/- 15	$V_\alpha11$	C A A TGTGCTGCT **E G A** G G Y K V GAGGGGGCTGGAGGCTATAAAACT	V F G GCTTTTGGA 12	$V_\beta3$	C A S TGTGCCAGC **S L N** R **G** Q D T Q AGTCTGAACAGGGGGCAAGACACCGAG	Y F G TACTTTGGG	2.5
+/- 8	$V_\alpha11$	C A A TGTGCTGCT **E A S** G S W Q L GAAGCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA 22	$V_\beta3$	C A S TGTGCCAGC **S L N** R **G** Q D T Q AGTCTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGG	2.5
+/- 1	$V_\alpha11$	C A A TGTGCTGCT **E P G** G N N K L GAGCCTGGAGGCAATAATAAGCTG	T F G ACTTTTGGT 56	$V_\beta3$	C A S TGTGCCAGC **S L N** W **G** Q D T Q AGTCTGAACTGGGCCCAAGACACCCAG	Y F G TACTTTGGG	2.5
MCC9	$V_\alpha11$	C A A TGTGCTGCT **E A S** S G Q K L GAAGCTTCAAGTGGCCAGAAGCTG	V F G GTTTTTGGC 16	$V_\beta3$	C A S TGTGCCAGC **S L N** R **A** N S D Y AGTCTGAACAAGGCAAACTCCGACTAC	T F G ACCTTCGGC	1.2
MCC11	$V_\alpha11$	C A A TGTGCTGCT **E S** T S S G Q K L GAGTCGACTTCAAGTGGCCAGAAGCTG	V F G GTTTTTGGC 16	$V_\beta16$	C A S TGTGCCAGC **S L D** T N Y A E Q AGCTTAGACACTAACTATGCTGAGCAG	F F G TTCTTCGGA	2.1
MCC25	$V_\alpha11$	C A A TGTGCTGCT **E A S** G G S N A K L GAGGCGTCTGGAGGAAGCAATGCAAAGCTA	T F G ACCTTCGGG 42	$V_\beta3$	C A S TGTGCCAGC **S L N** W **G** G G E Q AGTCTAACCTGGGGGGCGGAGAACAG	Y F G TACTTTGGG	2.6
MCC2	$V_\alpha11$	C A A TGTGCTGCT **E A** T G G Y K V GAGGCCACTGGAGGCTATAAAGTG	V F G GTGTTTGGA 12	$V_\beta3$	C A S TGTGCCAGC **S L N** R **G** Q D T Q AGTCTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGG	2.5
MCC14	$V_\alpha10$	C A L TGTGCTTTG D L N S N N R I GACCTTAATAGCAATAACAGAATC	F F G TTCTTTGGT 31	$V_\beta9$	C A S TGTGCTAGC S R D W G N T G Q L AGTAGAGATTGGGGAAACACCGGGCAGCTC	Y F G TACTTTGGT	2.2
MCC6				$V_\beta6$	C A S TGTGCCAGC S M W G A V N Y A E Q AGTATGTGGGGGGCGGTTAACTATGCTGAGCAG	F F G TTCTTCGGA	2.1
MCC8				$V_\beta3$	C A S TGTGCCAGC S L G T **G** R G Q D T Q AGTCTGGGGACTGGGAGGGGCCAAGACACCCAG	Y F G TACTTTGGG	2.5
99E29.1	$V_\alpha11$	C A A TGTGCTGCT **E S S** S F S K L GAGTCCTCCTCCTTCAGCAAGCTG	V F G GTGTTTGGG 50	$V_\beta1$	C A S TGTGCCAGC S G D W G G P F A E Q AGCCAAGACTGGGGGGGCCCCTTTGCTGAGCAG	Y F G TACTTTGGG	2.1
99E29.2	$V_\alpha8$	C A L TGTGCTCTG R G N M G Y K L AGGGGCAACATGGGCTACAAACTT	T F G ACCTTCGGG 9	$V_\beta8.2$	C A S TGTGCCAGC G D A Q G S A E T L GGTGATGCGCAGGGGAGTGCAGAAACCCTG	Y F G TATTTTGGC	2.3
99E18/5	$V_\alpha8$	C A L TGTGCTCTG S P Q N N Y A Q G L AGTCCCCAAAATAACTATGCCCAGGGATTC	T F G ACCTTCGGT 26	$V_\beta8.2$	C A S TGTGCCAGC G E S G G L E T L GGTGAATCAGGGGGCTTAGAAACGCTG	Y F G TATTTTGGC	2.3
99EC1	$V_\alpha11$	C A A TGTGCTGCC T T **S** G G N Y K P ACTACCTCAGGAGGAAACTACAAACCT	T F G ACGTTTGGG 6	$V_\beta8.3$	C A S TGTGCCAGC S G G Q A D T Q AGTGGGGGGCAGGCAGACACCCAG	Y F G TACTTTGGG	2.5
99EC13	$V_\alpha11$	C A A TGTGCTGCT **E A A** S S G Q K L GAGGCAACTTCAAGTGGCCAGAAGCTG	V F G GTTTTTGGC 16	$V_\beta16$	C A S TGTGCCAGC S A G T V Y A E Q AGCGCCGGGACAGTCTATGCTGAGCAG	F F G TTCTTCGGA	2.1
99E4C	$V_\alpha1$	C A A TGTGCAGCA S K T S G S W Q L AGCAAAACTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA 22	$V_\beta3$	C A S TGTGCCAGC S R G T **G** R G Q D T Q AGTCGCGGGACTGGGCGAGGCCAAGACACCCAG	Y F G TACTTTGGG	2.5
Hb7	$V_\alpha11$	C A A TGTGCTGCT **E A S** N T N K V GAGTCGACTTCAAGTGGCCAGAAG	V F G GTCTTTGGA 34	$V_\beta3$	C A S TGTGCCAGC S L **N** W **G** Q D T Q AGTCTCAACTGGGGGCAAGACACCCAG	Y F G TACTTTGGG	2.5
Hb2				$V_\beta8.2$	C A S TGTGCCAGC S G A R G L S N E R L AGCGGTGCCAGGGGCCTTTCCAACGAAAGATTA	F F G TTTTTCGGT	1.4
HB50	$V_\alpha11$	C A A TGTGCTGCT **E A S** S G Q K L GAAGCTTCAAGTGGCCAGAAGCTG	V F G GTTTTTGGC 16	$V_\beta14$	C A W TGTGCCTGG S L **N** R **A** Q D T Q AGTCTTAACAGGGCCCAAGACACCCAG	Y F G TACTTTGGT	2.5
HB60	$V_\alpha11$	C A A TGTGCTGCT **E P G** G N Y K P GAGCCAGGAGGAAACTACAAACCT	T F G ACGTTTGGG 6	$V_\beta16$	C A S TGTGCCAGC S S G T G V T E Q AGCTCCGGGACTGGGGTAACTGAGCAG	F F G TTCTTGGGA	2.1
HB70				$V_\beta1$	C A S TGTGCCAGC S Q Y R Y E Q AGCCAATACAGGTATGAACAG	Y F G TAGTTCGGT	2.6

Fig. 5. TCRα and TCRβ junctional region sequences for the panel of T hybridomas. Sequences were determined as in (*36*); J_α nomenclature is according to (*37*). Canonical amino acids for the response to MCC(88–103) are boxed.

neopeptide in the thymus provides a new approach for exploring the role of peptide in the positive selection of T cells. This strategy inverts the traditional one of starting with a T cell displaying a particular TCR and then attempting to define the requirements for its selection; rather, it begins with expression of a new peptide and permits one to study the T cells naturally selected on it. Our data show that the peptide sequence influences the sequence of the TCRs on selected cells, significant and systematic variations resulting from single-residue changes at putative TCR-contact points. The relation between selecting peptide and selected TCR shows significant, but not complete, two-way degeneracy, analogous to what is seen with the responses of mature T cells. Taken together, these observations support the hypothesis that positive selection involves direct recognition of peptide features, but they do not entirely rule out the possibility that peptide plays primarily a structural role, its precise sequence impinging on the process when it leads to steric hindrance of the TCR (*12*).

REFERENCES AND NOTES

1. H. von Boehmer, *Cell* **76**, 219 (1994); G. J. V. Nossal, *ibid.*, p. 229.
2. P. G. Ashton-Rickardt, L. Van Kaer, T. N. M. Schumacher, H. L. Ploegh, S. Tonegawa, *ibid.* **73**, 1041 (1993).
3. P. G. Ashton-Rickardt *et al.*, *ibid.* **76**, 651 (1994).
4. K. A. Hogquist, M. A. Gavin, M. J. Bevan, *J. Exp. Med.* **177**, 1469 (1993).
5. K. A. Hogquist *et al.*, *Cell* **76**, 17 (1994).
6. S. C. Jameson, K. A. Hogquist, M. J. Bevan, *Nature* **369**, 750 (1994).
7. K. A. Hogquist, S. C. Jameson, M. J. Bevan, *Immunity* **3**, 79 (1995).
8. E. Sebzda *et al.*, *Science* **263**, 1615 (1994).
9. E. Sebzda *et al.*, *J. Exp. Med.* **183**, 1093 (1996).
10. T. J. Pawlowski, M. D. Singleton, D. Y. Loh, R. Berg, U. D. Staerz, *Eur. J. Immunol.* **26**, 851 (1996).
11. H. M. Van Santen *et al.*, *J. Exp. Med.* **181**, 787 (1995).
12. T. N. M. Schumacher and H. L. Ploegh, *Immunity* **1**, 721 (1994).
13. L. Ignatowicz, J. Kappler, P. Marrack, *Cell* **84**, 521 (1996).
14. T. Miyazaki *et al.*, *ibid.*, p. 531.
15. W. D. Martin *et al.*, *ibid.*, p. 543; W.-P. Fung-Leung *et al.*, *Science* **271**, 1278 (1996).
16. R. Rooke, C. Benoist, D. Mathis, in preparation.
17. R. N. Germain, *Cell* **76**, 287 (1994).
18. S. Viville *et al.*, *ibid.***72**, 635 (1993). $Ii^{-/-}$ mice came from a backcross of the Ii null mutation onto the B10.BR background for three to five generations. Heterozygote controls were littermates or from parallel crosses to B10.BR.
19. S. Sanderson, K. Frauwirth, N. Shastri, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7217 (1995).
20. N. Nakano, unpublished results.
21. J. Kaye, G. Kersh, I. Engel, S. M. Hedrick, *Semin. Immunol.* **3**, 269 (1991); J. L. Jorgensen, P. A. Reay, E. W. Ehrich, M. M. Davis, *Annu. Rev. Immunol.* **10**, 835 (1992).
22. L. J. Berg, B. Fazekas de St. Groth, A. M. Pullen, M. M. Davis, *Nature* **340**, 559 (1989); S. Oehen, L. Feng, Y. Xia, C. D. Surh, S. M. Hedrick, *J. Exp. Med.* **183**, 2617 (1996).
23. S. M. Hedrick *et al.*, *Science* **239**, 1541 (1988); M. Cochet, *Eur. J. Immunol.* **22**, 2639 (1992); M. G. McHeyzer-Williams, M. M. Davis, *Science* **268**, 106 (1995).
24. S. Tourne, N. Nakano, S. Viville, C. Benoist, D. Mathis, *Eur. J. Immunol.* **25**, 1851 (1995).
25. J. J. Jorgensen, U. Esser, B. Fazekas de St. Groth, P. Z. Reay, M. M. Davis, *Nature* **355**, 224 (1992).
26. For the lymph node proliferation assays, 100 μg of peptide was injected with complete Freund's adjuvant into one footpad. Ten days later, draining popliteal lymph node cells were cultured in round-bottom 96-well plates (3×10^5 per well) with or without peptide for 72 hours. The cells were pulsed with 1 μCi of [^{3}H]thymidine for the last 18 hours of culture. Peptides used include MCC(88–103) (ANERADLIAYLKQATK) and its variants at position 99 or 102, Hb(67–76) (VITAFNEGJK), λCI(12–26) (LEDARRLKAIYEKKK), and RNase(90–105) (SKYPNCAYKTTQANKH), all synthesized by FMOC (fluorenyl methoxycarbonyl) chemistry and purified by reversed-phase high-performance liquid chromatography.
27. H.-E. Liang, C.-C. Chen, D.-L. Chou, M.-Z. Lai, *Eur. J. Immunol.* **24**, 1604 (1994).
28. P. J. Fink, M. J. Blair, L. A. Matis, S. M. Hedrick, *J. Exp. Med.* **172**, 139 (1990).
29. S. Tourne, T. Miyazaki, C. Benoist, D. Mathis, in preparation.
30. J. L. Casanova and J. L. Maryanski, *Immunol. Today* **14**, 391 (1993).
31. N. K. Nanda, K. K. Arzoo, H. M. Geysen, A. Sette, E. E. Sercarz, *J. Exp. Med.* **182**, 531 (1995); B. D. Evavold, J. Sloan-Lancaster, K. J. Wilson, J. B. Rothbard, P. Allen, *Immunity* **2**, 655 (1995); K. W. Wucherpfenning and J. L. Strominger, *Cell* **80**, 695 (1995).
32. T hybridoma cells (3×10^4) were cultured with 3×10^5 spleen cells as APCs, with or without added peptide. After 24 hours, the supernatants were collected and tested for interleukin-2 (IL-2) by proliferation (assayed as [^{3}H]thymidine incorporation) of the IL-2–dependent CTLL cell line.
33. A detailed description of the adenovirus vectors will be published elsewhere (*16*). Briefly, the chimeric Ii cDNA fragments were constructed by polymerase chain reaction (PCR) mutagenesis followed by ligation and insertion into the Eco RI–Bgl II sites of pNV4. The resulting plasmids were linearized, then transfected together with the right-hand Cla I fragment of dl324 into 293 cells (which complement the E1A deficiency; American Type Culture Collection CRL1573). Viral plaques stemming from recombination between plasmid and truncated adenoviral sequences were selected, screened for the presence of the Ii cDNA in the correct conformation, and plaque-purified twice. Large viral stocks were prepared in liquid cultures of 293 cells, purified, and concentrated on CsCl gradients. Purified virus was dialyzed against 0.5 M NaCl, 20 mM tris-HCl (pH 7.8), and samples were stored at −80°C, titered by limiting dilution (*16*), and verified. Before injection, virus was diluted three times in RPMI medium. Ten microliters of this dilution were injected into each thymic lobe of anesthetized mice with a Hamilton syringe (*16*). The titers of all concentrated viral stocks ranged from 0.3×10^{12} to 1.0×10^{12} IU/ml.
34. Draining lymph node cells from immunized mice were restimulated in vitro with 0.3 or 1.0 μM MCC(88–103) peptide for 2 days and expanded with IL-2 (50 U/ml) for another day. Blasts were fused with $\alpha^-\beta^-$ BW5147 cells (1/2 ratio) and hybrids selected in hypoxanthine, aminopterin, thymidine (HAT) medium. Hybridomas were tested for reactivity to MCC peptide presented by B10.BR splenocytes (*32*) and were recloned by limiting dilution.
35. For the antagonist assays, the B cell lymphoma line CH27 was treated with mitomycin C (25 μg/ml) for 30 min, pulsed with subsaturating doses of MCC(88–103) peptide for 3 hours at 37°C, and washed three times. These stimulators (3×10^4 per well) were cultured with the T cell hybridomas or clones (3×10^4 per well) in the presence of various doses of putative antagonist peptides. Hybridoma stimulation was measured as IL-2 production (*32*). Direct proliferation was measured for the T cell clone upon pulsing with 1 μCi of [^{3}H]thymidine for 18 hours after 48-hour incubation.
36. TCR sequences were determined from cytoplasmic RNA essentially as described [S. Candeias *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6167 (1991)].
37. B. F. Koop *et al.*, *Genomics* **19**, 478 (1994).

38. We thank M. Perricaudet for early help on this project; P. Allen for peptides; P. Marchal for much of the sequencing; M. Gilbert and C. Ebel for cells; F. Fischer, W. Magnant, and the staff of the Centre de Dévelopement des Techniques Avancées–CNRS for maintaining the mice; and P. Gerber for assistance. Supported by institutional funds from the INSERM, the CNRS, the Centre Hospitalier Universitaire Régional, Bristol Myers–Squibb, and by grants to D.M. and C.B. from the Association pour la Recherche sur le Cancer (ARC) and the Human Frontier Science Program. N.N. and R.R. were supported by fellowships from the ARC, CNRS, and Ligue Nationale contre le Cancer (LNCC), and the LNCC and Canadian Medical Research Council, respectively.

30 September 1996; accepted 3 December 1996

TECHNICAL COMMENTS

Comparative Rates of Development in *Monodelphis* and *Didelphis*

Timothy Rowe (*1*) presents a provocative hypothesis on the coevolution of the mammalian middle ear and neocortex, but there is a problem with the data presented in support of his hypothesis of a relation between brain growth and the detachment of the ear ossicles. Throughout the article, Rowe discusses the "didelphid" condition. Readers unfamiliar with the literature cited may not realize that in order to define the didelphid condition, Rowe combines data on *Didelphis* from the literature with his data on *Monodelphis* without acknowledging the differing rates of development in the two taxa. The two animals, although both didelphids, have different rates of postnatal growth and maturation. For example, in *Monodelphis*, the young first come off the teat at day 12, in *Didelphis* it is not until day 48 (*2*); in *Monodelphis* the young are weaned at day 50, in *Didelphis* it is after day 100 (*2*); in *Monodelphis* the auditory ossicles begin ossification on day 11 (*3*), in *Didelphis* it is during week 6 (*4*). We do not have information on the differences in timing of the specific events discussed by Rowe, but most information suggests that any given event will occur 2 to 4 weeks later in *Didelphis* than in *Monodelphis*.

The inappropriate combination of data occurs at multiple points in Rowe's report