

addition of 50 μ l 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 $\times$ 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 μ g), 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 antiserum): T. Perney, Rutgers University.

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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-phosphonopivalic acid and the fast component was abolished by 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 10 μ M).

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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 μ M. Thus, Ii is an effective "escort" of neopeptides joined to its COOH-terminus, consistent with previous experiments (19, 20).

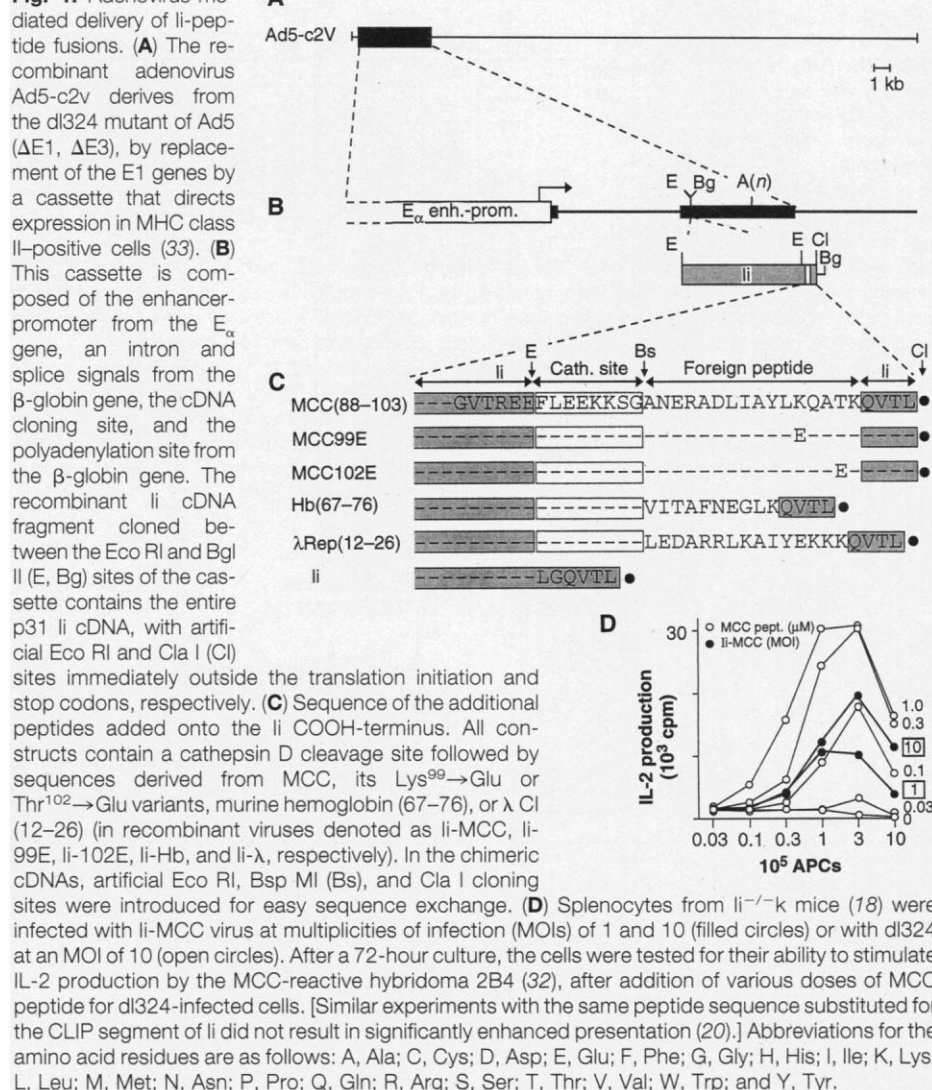
We used this system to study the positive selection of T cells capable of responding to the MCC(88–103) peptide in the context of E^k . The advantages of working with the anti-MCC response are that it is well characterized (21), its evaluation is facilitated by many existing reagents (22), it exhibits 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, $Ii^{-/-}$ 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. $Ii^{-/-}$ 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 chal-

lenged 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 $Ii^{-/-}$ 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 $V_\beta 3$, 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.



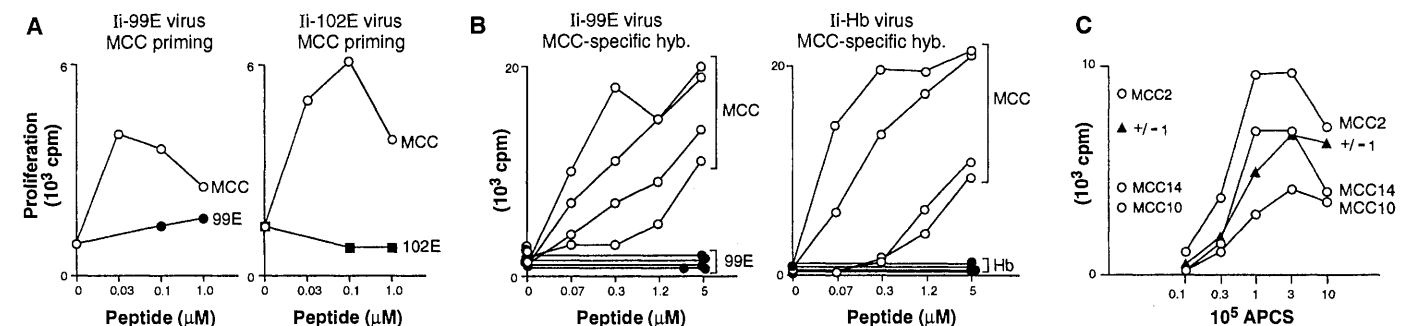
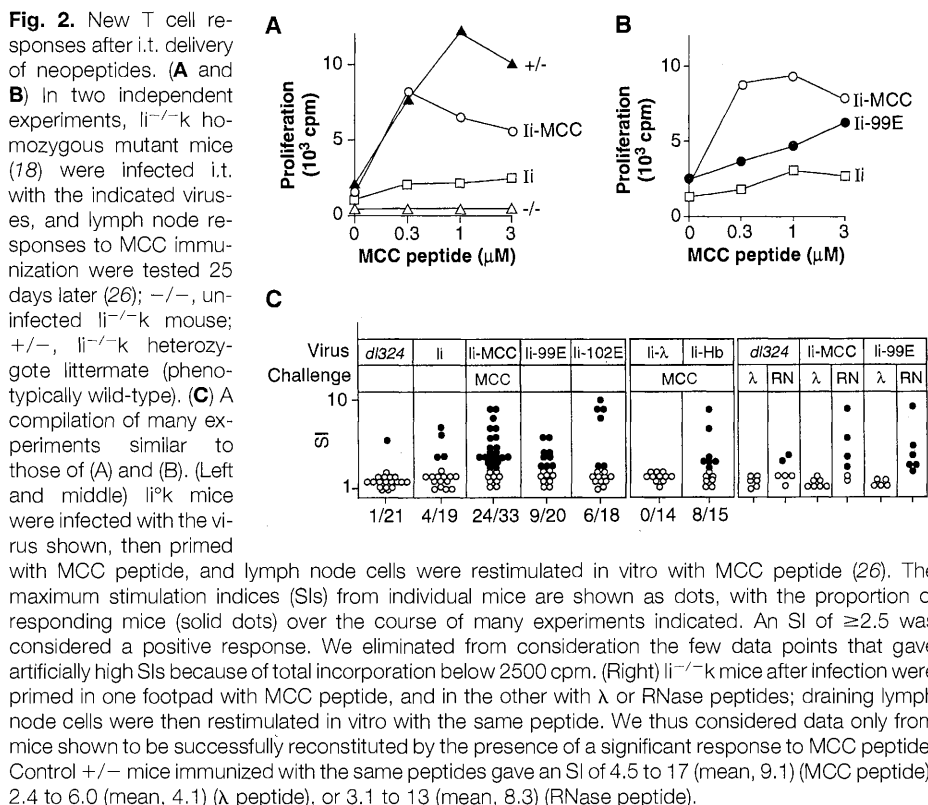
these two elicited responses that depended most critically on expression of Ii by APCs (20). Infection with either the Ii-MCC or the Ii-99E virus resulted in a response to the RNase peptide, but not to the λ peptide, in most animals. Thus, the delivery of neopeptides to thymic stromal cells has broad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



mal 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 μ M MCC peptide is represented by isolated dots.

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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 li-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 li-MCC virus rarely showed cross-reactivity, whereas those from animals injected with the li-99E virus frequently responded to MCC variants at position 102. Hybridomas from animals injected with the li-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 li-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 li-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 li alone (20). The hybridomas from animals injected i.t. with the li-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_{α} s and V_{β} s, greater variation in CDR-3 lengths, and alternate amino acids at conserved positions. The TCRs on hybridomas from animals injected with the li-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 li-Hb virus were again different: V_{α} usage was enriched in $V_{\alpha}11$ s with canonical CDR-3s; V_{β} usage was variable al-

though 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 li-MCC fusion protein promoted a

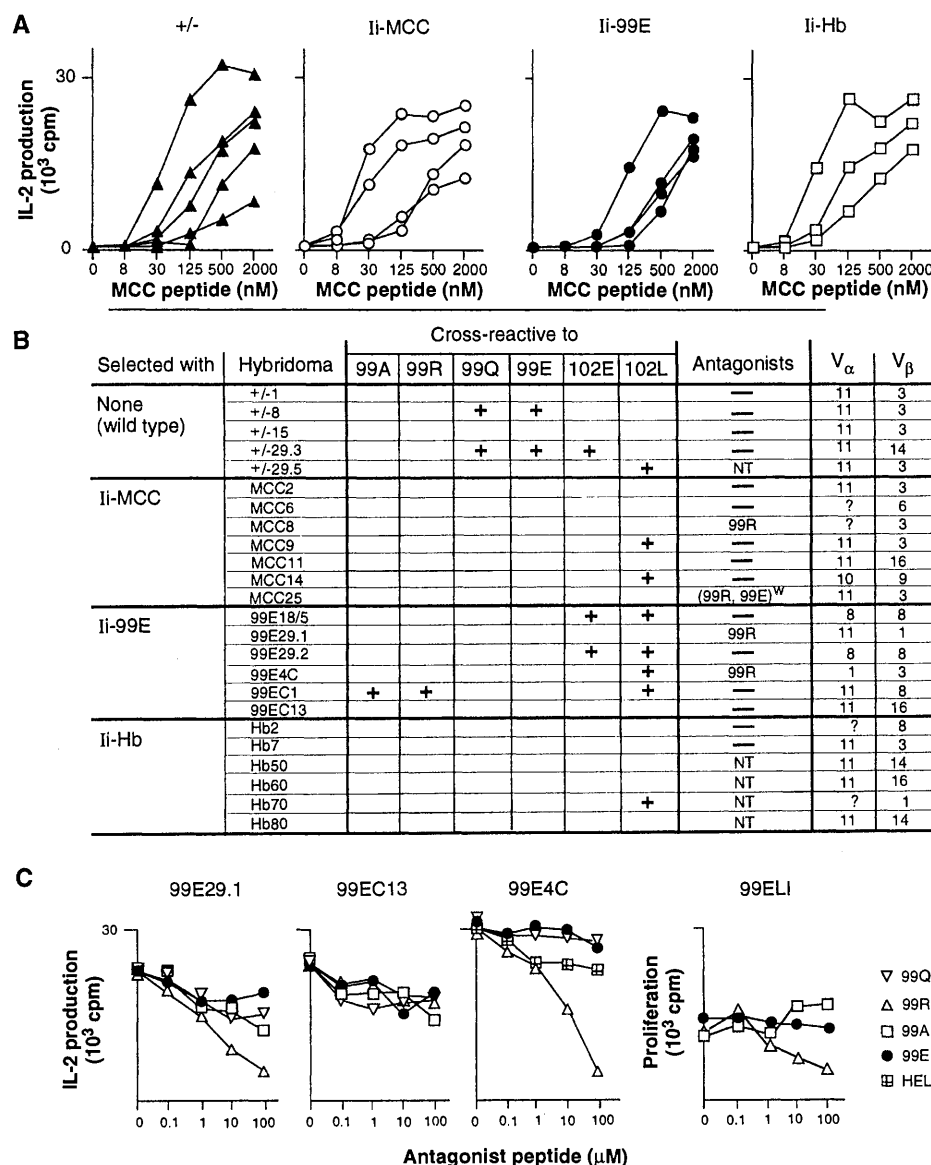


Fig. 4. Properties of hybridomas derived from mice expressing different neopeptides. Sets of MCC-reactive T hybridomas were isolated over the course of five independent fusions, originating from wild-type heterozygotes or from li^{+/+}-k mice infected i.t. with li-MCC, li-99E, or li-Hb, as shown (34). **(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 μ M); 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 TCR β sequencing [?, not determined (not $V_{\alpha}11$, 8, or 3)]. **(C)** Typical antagonist assays (35) for four cloned T cells derived from li-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 li-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 li-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 li-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 li-MCC-injected animals had overlapping sensitivities to MCC. In addition, the V_{β} usage of the $V_{\beta}3$ -negative MCC-selected hybridomas differed markedly from that of B10.BR mice injected from birth with a bacterial superantigen that deletes $V_{\beta}3$: heterogeneous in the former, almost solely $V_{\beta}8$ 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 spe-

cific 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 $V_{\alpha}11+V_{\beta}3$ 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 $V_{\alpha}11$ TCRs in our hybridomas) but that repertoire selection must be the driving force. We hypothesize that the canonical $V_{\alpha}11+V_{\beta}3$ receptors reflect selection by a dominant MHC-peptide complex, absent in li-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

Fig. 5. TCR α and TCR β 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.

Hybridoma	V_{α}	CDR3	J_{α}	V_{β}	CDR3	J_{β}
+/- 29-3	$V_{\alpha}11$	C A A [E] A [S] G S W Q L TGTGCTGCT GAGGCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA 22	$V_{\beta}14$	C A W [S] L [N] W [Q] Q D T Q TGTGCCCTGG AGCTGAALTTGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
+/- 15	$V_{\alpha}11$	C A A [E] G A [G] G Y K V TGTGCTGCT GAGGGGCTGGAGGCTATATAAAT	V F G GCTTTTGGG 12	$V_{\beta}3$	C A S [S] L [N] R [G] Q D T Q TGTGCCAGC AGCTTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
+/- 8	$V_{\alpha}11$	C A A [E] A [S] G S W Q L TGTGCTGCT GAGGCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA 22	$V_{\beta}3$	C A S [S] L [N] R [G] Q D T Q TGTGCCAGC AGCTTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
+/- 1	$V_{\alpha}11$	C A A [E] P [G] G N N K L TGTGCTGCT GAGGCTTGGAGCAATAAAGCTG	T F G ACTTTTGGT 56	$V_{\beta}3$	C A S [S] L [N] W [Q] Q D T Q TGTGCCAGC AGCTTGAALTTGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
MCC9	$V_{\alpha}11$	C A A [E] A [S] S G Q K L TGTGCTGCT GAGGCTTCTGGCAGCTGGCAACTC	V F G GTTTTGGC 16	$V_{\beta}3$	C A S [S] L [N] R [A] N S D Y TGTGCCAGC AGCTTGAACAGGGGGCAAGACACCCAG	T F G ACCTTGGC 1.2
MCC11	$V_{\alpha}11$	C A A [E] S T S S G Q K L TGTGCTGCT GAGTGCAGCTTCAAGTGCCAGAGCTG	V F G GTTTTGGC 16	$V_{\beta}16$	C A S [S] L D T N Y A E Q TGTGCCAGC AGCTTGAACAGGGGGCAAGACACCCAG	F F G TCTTTCGGA 2.1
MCC25	$V_{\alpha}11$	C A A [E] A [S] G S N A K L TGTGCTGCT GAGGCTTCTGGAGGCAAGCAAGCTA	T F G ACCTTGGG 42	$V_{\beta}3$	C A S [S] L [N] W [Q] G G E Q TGTGCCAGC AGCTTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.6
MCC2	$V_{\alpha}11$	C A A [E] A T G G Y K V TGTGCTGCT GAGGCTTCTGGAGGCAAGCAAGCTA	V F G GTGTTGGA 12	$V_{\beta}3$	C A S [S] L [N] R [G] Q D T Q TGTGCCAGC AGCTTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
MCC14	$V_{\alpha}10$	C A L D L N S N N R I TGTGCTTGG GACCTTAATAGCAATAACAGATC	F F G TCTTTGGT 31	$V_{\beta}9$	C A S S R D W G N T G Q L TGTGCTAGC AGTGAAGATTTGGGGAACACCGGAGCTC	Y F G TACTTTGGT 2.2
MCC6				$V_{\beta}6$	C A S S M W G A V N Y A E Q TGTGCCAGC AGTATGTTGGGGGGCGGTTAATCTAGCTGAGCAG	F F G TCTTTCGGA 2.1
MCC8				$V_{\beta}3$	C A S [S] L G T [G] R G Q D T Q TGTGCCAGC AGCTTGGGAGCTGGGAGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
99E29.1	$V_{\alpha}11$	C A A [E] S [S] S F S K L TGTGCTGCT GAGTCTCTCTCCCTTACAGCAAGCTG	V F G GTGTTGGG 50	$V_{\beta}1$	C A S S G D W G G P F A E Q TGTGCCAGC AGCCCAAGAGCTGGGGGGGCGCTTTGCTGAGCAG	Y F G TACTTTGGG 2.1
99E29.2	$V_{\alpha}8$	C A L R G N M G Y K L TGTGCTCTG AGGGGCAACATGGGCTACAAATC	T F G ACCTTGGG 9	$V_{\beta}8.2$	C A S G D A Q G S A E T L TGTGCCAGC GGTGATGCGCAGGGGAGTGCAGAAACCTG	Y F G TATTTTGGC 2.3
99E18/5	$V_{\alpha}8$	C A L S P Q N N Y A Q G L TGTGCTCTG AGTCCCAAAATCAATATGCCAGGATTC	T F G ACCTTGGT 26	$V_{\beta}8.2$	C A S S G G L E T TGTGCCAGC AGTCTGAACAGGGGGCTTGAAGACGCTG	Y F G TACTTTGGG 2.3
99EC1	$V_{\alpha}11$	C A A [E] A T T [G] G N Y K P TGTGCTGCT ACTACCTTGGAGGAACTACAACT	T F G ACGTTGGG 6	$V_{\beta}8.3$	C A S S G G Q A D T Q TGTGCCAGC AGTGGGGGGGAGGAGACACCCAG	Y F G TACTTTGGG 2.5
99EC13	$V_{\alpha}11$	C A A [E] A [S] S G Q K L TGTGCTGCT GAGGCAATTTCAAGTGCCAGAGCTG	V F G GTTTTGGC 16	$V_{\beta}16$	C A S S A G T V Y A E Q TGTGCCAGC AGCCCGGAGACAGCTATGCTAGCAGCAG	F F G TCTTTCGGA 2.1
99E4C	$V_{\alpha}1$	C A A S K T S G S W Q L TGTGCCAGC AGCAAAAGCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA 22	$V_{\beta}3$	C A S S R G T [G] R G Q D T Q TGTGCCAGC AGTCCGGAGAGCTGGGAGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
Hb7	$V_{\alpha}11$	C A A [E] A [S] N T N K V TGTGCTGCT GAGTGCAGCTTCAAGTGCCAGAGG	V F G GTCTTTGGA 34	$V_{\beta}3$	C A S [S] L [N] W [Q] Q D T Q TGTGCCAGC AGCTTGAALTTGGGGGCAAGACACCCAG	Y F G TACTTTGGG 2.5
Hb2				$V_{\beta}8.2$	C A S S G A R G L S N E R L TGTGCCAGC AGCCGTGCGCAGGGGCTTTTCAAGCAAGATTA	F F G TCTTTCGCT 1.4
HB50	$V_{\alpha}11$	C A A [E] A [S] S G Q K L TGTGCTGCT GAGGCTTCTGGAGGCAAGCAAGCTG	V F G GTTTTGGC 16	$V_{\beta}14$	C A W [S] L [N] R [A] Q D T Q TGTGCCCTGG AGCTTGAACAGGGGGCAAGACACCCAG	Y F G TACTTTGGT 2.5
HB60	$V_{\alpha}11$	C A A [E] P [G] G N Y K P TGTGCTGCT GAGGCTTGGAGGAACTACAACT	T F G ACGTTGGG 6	$V_{\beta}16$	C A S S S G T G V T E Q TGTGCCAGC AGCTCCGGAGCTGGGTAAGTACGAGCAG	F F G TCTTTCGGA 2.1
HB70				$V_{\beta}1$	C A S S Q Y R Y E Q TGTGCCAGC AGCCCAATACAGGTATGAACAG	Y F G TAGTTCGCT 2.6

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).

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- For the lymph node proliferation assays, 100 μ g 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 μ Ci 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) (VI-TAFNEGJK), λ CI(12–26) (LEDARRLKAIYEKKK), and RNase(90–105) (SKYPNCAYKTTQANKH), all synthesized by FMOC (fluorenyl methoxycarbonyl) chemistry and purified by reversed-phase high-performance liquid chromatography.
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- 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.
- A detailed description of the adenovirus vectors will be published elsewhere (16). Briefly, the chimeric l^{i} 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 Δ 324 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 l^{i} 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.
- Draining lymph node cells from immunized mice were restimulated in vitro with 0.3 or 1.0 μ M MCC(88–103) peptide for 2 days and expanded with IL-2 (50 U/ml) for another day. Blasts were fused with α - β -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.
- For the antagonist assays, the B cell lymphoma line CH27 was treated with mitomycin C (25 μ g/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 μ Ci of [3 H]thymidine for 18 hours after 48-hour incubation.
- TCR sequences were determined from cytoplasmic RNA essentially as described [S. Candeias *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6167 (1991)].
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- 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 Développement 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 *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