



Comparative Rates of Development in Monodelphis and Didelphis

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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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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 × 10⁵ per well) with or without peptide for 72 hours. The cells were pulsed with 1 µCi of [³H]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), λCl(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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32. T hybridoma cells (3 × 10⁴) were cultured with 3 × 10⁵ 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 [³H]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 *li* 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 *li* 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 × 10¹² to 1.0 × 10¹² 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 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.
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 × 10⁴ per well) were cultured with the T cell hybridomas or clones (3 × 10⁴ 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 [³H]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)].
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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 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.

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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

(1); however, the problem is particularly critical in figure 4, which provides the primary data to suggest a relation between the growth of the brain and the detachment of the ossicles. In this figure, Rowe superimposes his data on the growth of the ectotympanic and dentary bones and the date of the detachment of the auditory ossicles in *Monodelphis* on data on brain growth in *Didelphis* presented by Ulinski (5). He does not correct for the differing rates of development; instead, the two data sets are combined. This is equivalent to taking one set of measurements on a domestic cat and another on a tiger and, without correction for size or rates of development, summarizing the "felid" pattern. The auditory ossicles do not detach from Meckel's cartilage at day 21 in *Didelphis* because at this time there is no jaw condyle nor is there ossification of any ossicle (4). Further, all evidence suggests that at 20 days after birth the brain is far more advanced in *Monodelphis* than in a 20-day *Didelphis* pouch young (6). If Rowe is to argue a relation between the timing of events in development, he must either compare data derived from a single species or, at the least, correct for the differing rates of development in two very different species.

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Response: Do *Didelphis* and *Monodelphis* really have differing rates of growth? In answering this question, care must be taken to distinguish between rates of growth and rates of maturation because the two are broadly correlated but are not strictly coupled throughout ontogeny (1).

Didelphis and *Monodelphis* undoubtedly have different growth rates. These closely

related didelphid marsupials (2) have similar life-spans of 2 to 3 years in the wild, yet *Didelphis* reaches two to three times the adult size of *Monodelphis* (3). This accords with the observation that *Didelphis* young remain attached to the teat longer and are weaned much later than *Monodelphis* young.

Do rates of maturation also differ? My observations on skeletal maturation in *Monodelphis* (4–5) agree closely with those of Smith (6) and van Nievelt, but we disagree on the timing of maturation events in *Didelphis*. Their statements about *Didelphis* are based on a study by Nesslinger (7), who examined only whole specimens that were cleared and stained for bone (alizarin). As histology shows, clearing and staining does not allow one to detect bone at its earliest stages in ontogeny. Nesslinger's specimens consisted of only road-killed and wild-caught *Didelphis*, so that chronological ages could only be approximated. More thorough studies on the embryology of *Didelphis* (8–11) were based on a collection of several hundred specimens raised by the Wistar Institute in the 1930s. Histological sectioning of individuals of known ages indicates that, insofar as the skeletons of *Monodelphis* (4–6, 12) and *Didelphis* (8–11) can be compared, they are virtually identical in timing of maturation.

For example, a synovial joint is present between the incus and malleus at birth in both *Didelphis* (10) and *Monodelphis* (12). Ossification of the ectotympanic has begun by the middle of the second day in both species. In *Didelphis* (10, p. 235)

at 7 days the mandible has a definite temporomandibular articulation . . . the mandibular condyle contains a larger condylar cartilage which has developed between the seventh and fifteenth day. It is rather large and is already undergoing some ossification . . .

just as in *Monodelphis* (5, 6, 12). Ossification of the malleus has begun in both *Monodelphis* (5) and *Didelphis* (10) by the end of the second week. By the third week the incudo-malleolar joint is well formed and enclosed in a fibrous joint capsule in both species. In the fourth week, about the time of detachment, the incudo-stapedial joint becomes well formed and also enclosed in a fibrous joint capsule in both species. Over the remainder of ontogeny, the bones of the auditory chain in the two didelphids share similar chronologies. My examination of the surviving materials from the Wistar col-

lection and other large North American skeletal collections of *Didelphis* substantiates these observations (5); I can find no support for the statement that "any given event will occur 2 to 4 weeks later in *Didelphis* than in *Monodelphis*." Although didelphid species have different growth rates, their chronologies of maturation are closely comparable.

Last, the relation that I described between the brain and middle ear (4, 5) is one of relative growth, not timing of maturation. The relative size of the adult brain varies over more than an order of magnitude among different mammalian species, hence mammals must have widely varying rates of brain growth (13). But the small middle ear ossicles are far less variable in size, their growth ceasing early in ontogeny as a constraint of their function in high-frequency audition. Repositioning of the auditory chain occurs in the wake of continued cerebral growth. Didelphids are among the least encephalized mammals and offer the most generalized examples of this relationship. The patterns of variability among other species are invariably superimposed upon a more general pattern of differential growth of the brain and middle ear bones that is common to all mammals.

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