carried out with BlastP [S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990)] v.1.3.11MP and the National Center for Biotechnology Information nonredundant peptide sequence database. The nucleotide sequence encoding *RPS2* has been deposited with GenBank (accession number U14158).
25. B. Kobe and J. Deisenhofer, *Nature* **366**, 751 (1993).
26. N. Suzuki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8711 (1990).
27. J. A. Lopez *et al.*, *ibid.* **84**, 5615 (1987).
28. T. Braun, P. R. Schofield, R. Sprengel, *EMBO J.* **10**, 1885 (1991).
29. D. S. Schneider, K. L. Hudson, T. Lin, K. V. Anderson, *Genes Dev.* **5**, 797 (1991).
30. Abbreviations for the amino acid residues are: 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. X indicates any amino acid.
31. R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.* **54**, 631 (1985).
32. T. Alber, *Curr. Opin. Gen. Dev.* **2**, 205 (1992).
33. A. Lupas, M. Van Dyke, J. Stock, *Science* **252**, 1162 (1991).
34. M. Saraste, P. R. Sibbald, A. Wittinghofer, *Trends Biochem. Sci.* **15**, 430 (1990).
35. S. C. Winans, R. A. Kerstetter, E. W. Nester, *J. Bacteriol.* **170**, 4047 (1988).
36. G. von Heijne, *J. Mol. Biol* **225**, 487 (1992).
37. S. Whitham *et al.*, *Cell*, in press.
38. S. Gibson and C. Somerville, in *Methods in* Arabidopsis *Research*, C. Koncz, N.-H. Chua, J. Schell, Eds. (World Scientific, London, 1992), pp. 119–143.
39. E. R. Ward and G. C. Jen, *Plant Mol. Biol.* **14**, 561 (1990); J. R. Ecker, *Methods* **1**, 186 (1990); E. Grill and C. Somerville, *Mol. Gen. Genet.* **226**, 484 (1991).
40. J. Leung *et al.*, *Science* **264**, 1448 (1994).
41. A. Konieczny and F. M. Ausubel, *Plant J.* **4**, 403 (1993).
42. C. Chang, J. L. Bowman, A. W. DeJohn, E. S. Lander, E. M. Meyerowitz, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6856 (1988).
43. J. D. G. Jones *et al.*, *Transgenic Res.* **1**, 285 (1992).
44. J. M. Cherry, S. W. Cartinhour, H. M. Goodman, *Plant Mol. Biol. Report.* **10**, 308 (1992).
45. M. J. Daniels, C. E. Barber, D. C. Turner, S. G. Cleary, M. K. Sawzyc, *J. Gen. Microbiol.* **130**, 2447 (1984).
46. A.F.B. and B.N.K. contributed equally to this work. We thank T. Tai for the cDNA library, G.-L. Yu and M. Mindrinos for the *rps2-101C* and *rps2-101N* mutants, and C. Lister and C. Dean for vector pCLD04541. We also thank M. Mindrinos, F. Katagiri, G.-L. Yu, F. Ausubel, and B. Baker for sharing results before publication and C. Dean for her support during construction of the YAC contig. Supported in part by grants from the U.S. Department of Energy (B.J.S.), C.N.R.S. (J.G.), and the American Cancer Society, California Division Postdoctoral Fellowship (B.N.K.).

25 July 1994; accepted 1 September 1994

Thymus-Neuroendocrine Interactions in Extrathymic T Cell Development

Jin Wang and John R. Klein*

Studies of the development of murine intestinal intraepithelial lymphocytes (IELs) have yielded markedly different results depending on the experimental system used. In athymic radiation chimeras, IELs consist of all subsets found in euthymic mice; adult mice that were athymic at birth have only IELs that are positive for T cell receptor γδ and CD8αα. These differences are resolved by the finding that administration of the neuropeptide thyrotropin-releasing hormone to adult mice thymectomized as neonates leads to the development of all IEL T cells. Thus, a neuroendocrine signal initiated by the thymus during fetal or neonatal life appears to be required for subsequent extrathymic maturation of gut αβ T cells.

There now is considerable evidence that some, or possibly all, murine intestinal IELs are T cells that have matured independent of the thymus (*1–4*), thereby constituting the largest group of peripheral extrathymic T cells. Yet, opinions differ sharply as to which IELs are thymus-dependent and which are thymus-independent (*5*, *6*); those differences primarily reflect the particular experimental system used—that is, whether mice were made athymic as adults (athymic radiation chimeras) (*2*, *3*) or whether they were athymic from birth (*4*, *6*). Understanding the basis for those differences is essential for determining which IELs are truly extrathymic T cells and for understanding which experimental system most accurately reflects the biology of the IELs in normal mice. In that context, several studies have recently reported that engraftment into neonatally athymic mice with fetal thymus or with thymus tissues contained in diffusion chambers (*6*) leads to the appearance of αβ T cells in the gut. Because in the latter experiments lymphocytes could not enter the engrafted thymus tissue, it was concluded that the thymus stroma directly influences the development of αβ T cells within the gut. As reported here, however, a similar outcome can be achieved by neurohormone supplementation in the absence of thymus engraftment, which suggests that although the thymus is involved in some stage of that process, it is not directly responsible for the development of gut αβ T cells.

We isolated IELs from adult athymic radiation chimeras and from adult neonatally thymectomized (NTX) mice (*7*) and compared them for expression of lymphocyte markers by flow cytometric analyses (*8*) to IELs from normal euthymic mice (that is, mice with normal thymuses). Because most TCRαβ, CD8αβ, and CD5 IELs express Thy-1 (*9*, *10*) and because those populations were of particular interest in our study, the hematopoietic origins of IELs in athymic chimeras were determined with irradiated CBA (Thy-1.2) congenic mice injected with bone marrow from AKR (Thy-1.1) mice. More than 90% of Thy-1$^+$ IELs in athymic chimeras expressed the Thy-1.1 allele, which indicates that they were donor-derived lymphocytes and that they were not residual host cells (Fig. 1). IELs from athymic chimeras (Fig. 2B) expressed all subsets that existed in euthymic mice (Fig. 2A). In those mice, IELs consisted of both TCRαβ$^+$CD5$^+$ cells and TCRγδ$^+$CD5$^-$ cells. Virtually all CD8β$^+$ IELs were Thy-1$^+$ cells, and both CD8αα and CD8αβ IELs were present. CD5 was expressed on some, though not all, TCRαβ$^+$ IELs; TCRγδ$^+$ IELs were primarily CD5$^-$. Consistent with findings from this (*2*) and other laboratories (*3*), T cells were absent in peripheral immune compartments outside the intestine. These findings indicate that despite a reduction in the proportion of some types of IELs in athymic chimeras (for example, CD8αβ$^+$ and Thy-1$^+$CD8β$^+$ cells) (Fig. 2B), all IEL subsets nonetheless developed in the absence of the thymus.

In contrast to IELs in euthymic mice and athymic chimeras, IELs in NTX mice consisted of only TCRγδ$^+$CD8αα$^+$ cells (Fig. 2C). Additionally, more than half of the IELs in NTX mice were devoid of markers of mature lymphocytes and, even among the TCRγδ$^+$ IELs, there was a reduction in the proportion of TCRγδ IELs in NTX mice compared to the number of IELs from athymic chimeras (Fig. 2). However, the most striking difference between IELs in athymic chimeras and those in NTX mice was the absence of TCRαβ, CD8αβ, Thy-1, and CD5 cells, a finding that was consistent in several IEL isolates from NTX mice. Thus, IELs in mice that are athymic at birth lack αβ T cells.

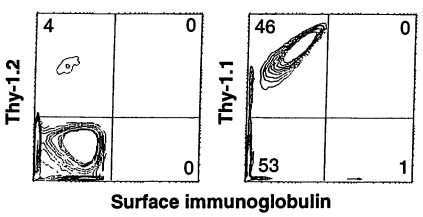

Fig. 1. Expression of Thy-1.1 and Thy-1.2 on IELs from CBA athymic radiation chimeras injected with AKR bone marrow (*7*).

Department of Biological Science and Mervin Bovaird Center for Studies in Molecular Biology and Biotechnology, University of Tulsa, Tulsa, OK 74104, USA.

*To whom correspondence should be addressed.

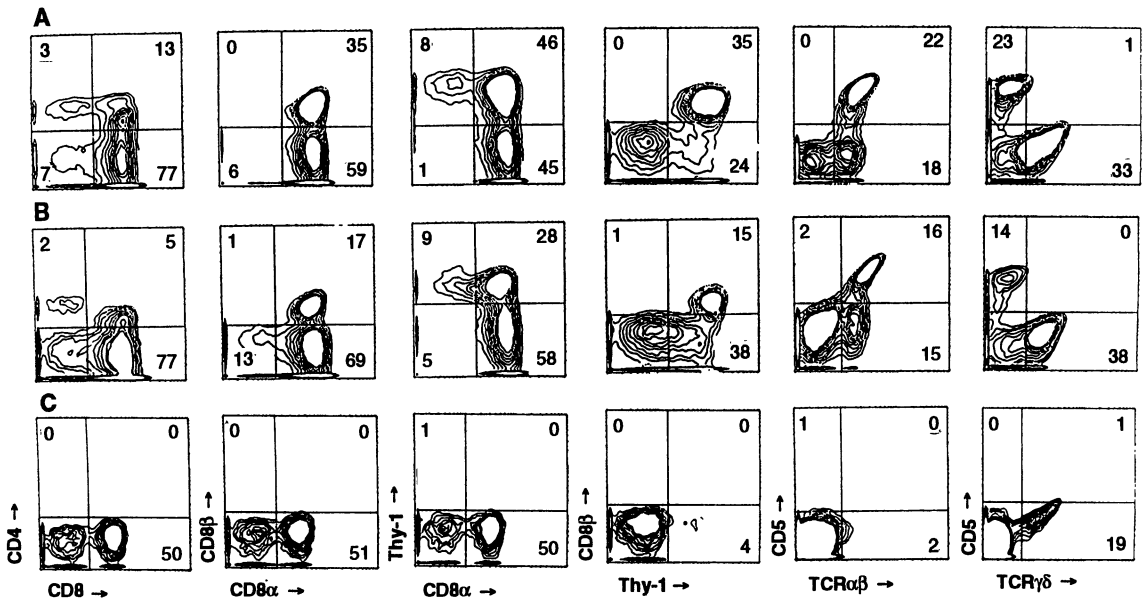

Fig. 2. Phenotypic analyses of gut IELs from (**A**) age-matched euthymic mice, (**B**) athymic radiation chimeras, and (**C**) NTX mice. Phenotypically, IELs from athymic chimeras are similar to IELs from euthymic mice, whereas IELs from NTX mice lack TCRαβ, CD8αβ, Thy-1, and CD5 subsets.

It is known that the hypothalamus-derived neuroendocrine hormone, thyrotropin-releasing hormone (TRH), and pituitary hormones that are regulated by TRH influence the development and function of the thymus-dependent branch of the lymphoid system, particularly during the fetal or neonatal period (*11–15*). As yet, however, nothing is known about the involvement of the neuroendocrine system in the maturation of gut T cells. Because athymic chimeras and NTX mice differed according to the time at which they were made athymic, we sought to determine whether the lack of αβ T cells in IELs from NTX mice was due to a disruption of the thymus-neuroendocrine axis as a result of thymectomy during an early phase of ontogeny.

Adult NTX mice, 8 to 10 weeks of age, were administered exogenous TRH (*16*), and their IELs were studied phenotypically 1 and 3 weeks after treatment. TRH was chosen because of its well-documented effects on T cell development and because of its ability to induce the release of other immunologically relevant neuroendocrine hormones (*11–15*). The IELs from adult NTX mice that had been treated with TRH for 1 week began to express subsets that were present in athymic chimeras and euthymic mice (Fig. 3A), but that were routinely absent in NTX mice. By 3 weeks after TRH treatment, there was an increase in NTX mice in the proportion of IELs expressing TCRαβ, CD8αβ, Thy-1, and CD5 (Fig. 3) compared to the number of IELs in age-matched untreated NTX mice (Figs. 2C). Those findings were consistent for several TRH-treated mice as well (Table 1). Moreover, the total number of recovered IELs also had increased significantly in TRH-treated mice: $(7.6 \pm 3.2) \times 10^6$ IELs per untreated mouse versus $(20.1 \pm 3.3) \times 10^6$ IELs per TRH-treated mouse. Because the effect of TRH treatment was primarily directed at intestinal T cells, as seen by the lack of splenic lymphocytes expressing CD4, CD8, TCRαβ, or TCRγδ (Table 1), and because TRH is not directly mitogenic for murine lymphocytes in vitro (*17*), the presence of TCRαβ T cells in the gut epithelium of TRH-treated mice was not a result of the expansion of small numbers of thymus-derived T cells. Rather, the absence of $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs in NTX mice appears to be directly related to a neuroendocrine deficiency in those mice.

These findings suggest that a neuroendocrine factor is important for the local development of gut αβ T cells and that a thymus-mediated signal is needed early in ontogeny. This explains why IELs from adult athymic chimeras bear all subsets found in euthymic mice, whereas IELs from NTX mice lack αβ T cells. Although the mode of action of TRH on the development of gut αβ T cells is not yet evident, the effect of TRH may be to induce the release of immunologically relevant pituitary hormones such as thyroid-stimulating hormone (TSH). In fact, gut T cell development also occurred efficiently in TSH-treated NTX mice (*18*). Moreover, other studies have documented neuroendocrine involvement in the mat-

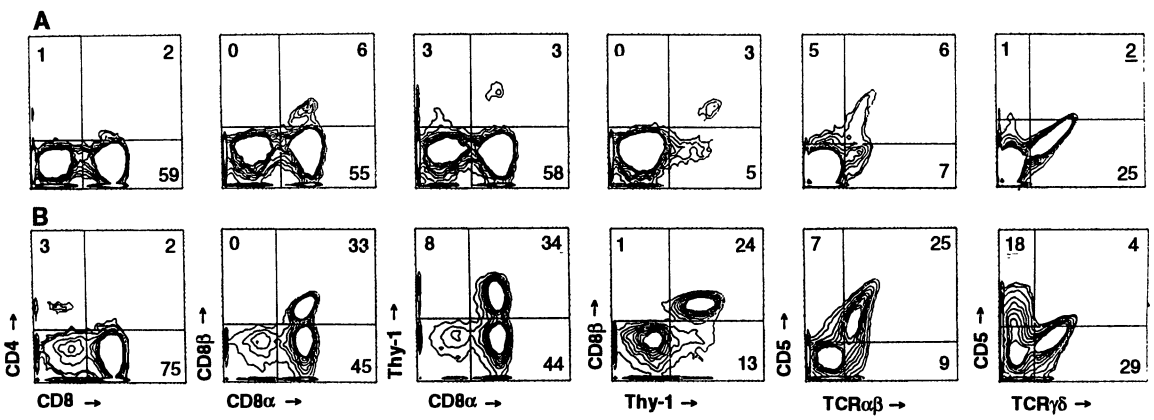

Fig. 3. Phenotypic analyses of IELs from NTX mice treated with TRH for (**A**) 1 week or (**B**) 3 weeks. Note the presence of TCRαβ, CD8αβ, Thy-1, and CD5 IEL subsets after TRH treatment in the absence of direct immune augmentation.

Table 1. Effect of TRH treatment on IEL development in NTX mice. The percentage of total lymphocytes represents the mean value ± SEM of staining of IELs from four to six mice per group, isolated, stained, and analyzed individually (*7*). ND, not determined.

Marker	Percentage of total lymphocytes			
	IELs		Spleen	
	Untreated mice	TRH-treated mice	Untreated mice	TRH-treated mice
Thy-1	4.0 ± 1.5	39.6 ± 8.7*	3.1 ± 2.4	5.0 ± 0.7
CD4	0.8 ± 0.3	2.1 ± 1.4	2.5 ± 0.3	2.6 ± 0.6
CD5	1.0 ± 1.1	24.6 ± 8.2*	ND	ND
CD8	51.3 ± 2.7	71.6 ± 6.6	1.2 ± 1.0	1.6 ± 0.5
CD8αα	47.6 ± 5.2	43.0 ± 4.4	ND	ND
CD8αβ	0.9 ± 0.8	37.3 ± 5.1*	ND	ND
TCRγδ	17.5 ± 4.3	32.2 ± 2.5*	<1	<1
TCRαβ	1.5 ± 0.7	29.6 ± 3.9*	<1	<1

*Statistically significant difference ($P < 0.001$) compared to untreated NTX mice according to Student's t test for unpaired observations.

uration of the T cell lineage, and vice versa (*11–13*, *14*). Mice that are athymic at birth have deficiencies in hypothalamic or pituitary hormone production, which can be partially or completely restored by thymus implantation (*11*). In contrast, animals thymectomized as adults have minimal hormone impairment (*11*). Neuroendocrine hormones also have been shown to influence the maturation of the thymus epithelium (*14*). Given existing evidence for the role of the gut epithelium in antigen presentation and development (*19*), it is possible that the effect of neuroendocrine hormones is to render the gut epithelium suitable for the maturation of αβ T cells. Both positive and negative selection now have been shown to occur during the extrathymic development of $\alpha\beta^+$ IELs in athymic chimeras (*3*), and major histocompatibility complex–restricted IEL T cells have been reported (*10*, *20*). In contrast, there is growing evidence that at least some γδ T cells are not selected on self tissue antigens (*21*) or that they recognize antigens in a manner different from that of αβ T cells (*22*), which implies distinct requirements for αβ and γδ T cells during maturation.

Finally, it should be noted that gut T cell development did not occur in hormone-treated, congenitally athymic nude mice. Considering the extensive hypothalamic, pituitary, and thyroid defects that exist in nude mice (*23*), this is not necessarily surprising. Moreover, the lack of a response in nude mice may reflect the total absence of a thymus-derived signal, given that nude mice are athymic during fetal life, whereas NTX mice are not, thus further implying that the process needed to activate a neuroendocrine response occurs during the fetal stage. That possibility is compatible with reports that engraftment of thymus tissues into nude mice leads to normal IEL development (*6*), presumably by activating the entire thymus-neuroendocrine loop. Taken together, the findings reported here suggest a complex physiological network needed for extrathymic T cell maturation.

REFERENCES AND NOTES

1. H. Kiyono and J. R. McGhee, Eds., *Mucosal Immunology: Intraepithelial Lymphocytes (IELs)* (Raven, New York, 1994), pp. 1–195; G. Mayrhofer and R. J. Whately, *Int. Arch. Allergy Appl. Immunol.* **71**, 317 (1983); G. Mayrhofer, *Blood* **55**, 532 (1980); J. Klein, *J. Exp. Med.* **164**, 309 (1986).
2. R. L. Mosley, D. Styre, J. R. Klein, *J. Immunol.* **145**, 1369 (1990); M. Whetsell *et al.*, *Mol. Cell. Biol.* **11**, 5902 (1991); R. L. Mosley and J. R. Klein, *Transplantation* **53**, 868 (1992); D. Stickney *et al.*, *Mol. Immunol.* **30**, 813 (1993).
3. P. Poussier, P. Eduoard, C. Lee, M. Julius, *J. Exp. Med.* **176**, 187 (1992); P. Poussier, H. S. Teh, M. Julius, *ibid.* **178**, 1947 (1993).
4. J. L. Viney, T. T. MacDonald, P. J. Kilshaw, *Immunology* **66**, 583 (1989); D. Guy-Grand, N. Cerf-Bensussan, N. Malissen, C. Briottet, P. Vassalli, *J. Exp. Med.* **173**, 471 (1991); B. Rocha, P. Vassalli, D. Guy-Grand, *ibid.*, p. 483; A. Bandiera *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 43 (1991).
5. D. Guy-Grand and P. Vassalli, *Immunol. Res.* **10**, 296 (1991); L. Lefrancois, *Immunol. Today* **12**, 436 (1991); J. R. Klein and R. L. Mosley, *ibid.* **13**, 148 (1992); B. Rocha *et al.*, *ibid.*, p. 449; D. Guy-Grand, *Curr. Opin. Immunol.* **5**, 247 (1993); P. Poussier and M. Julius, *Annu. Rev. Immunol.* **12**, 521 (1994).
6. T. Lin *et al.*, *Eur. J. Immunol.* **23**, 1968 (1993); H. Kenai *et al.*, *ibid.*, p. 1818.
7. C57BL/6, CB6F1, CBA, and AKR mice, 8 to 10 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, ME. Construction of athymic chimeras was done according to procedures described elsewhere (*2*), except that day 15 to 16 fetal liver cells were used as a source of hematopoietic stem cells. Briefly, adult mice, 8 to 10 weeks of age, were anesthetized by intraperitoneal injection of Avertin [10 g of tribromoethyl alcohol in 10 ml of *tert*-amyl alcohol (Aldrich), diluted to 2.5% (v/v) in phosphate-buffered saline]. Mice were thymectomized by aspiration of the thymus through a trans-sternal incision. Two to three weeks later, mice were irradiated with 10 Gy (units of absorbed dose of ionizing radiation) from a ^{137}Cs source followed by intravenous injection of 10^7 fetal liver cells. Athymic chimeras were used 2 to 4 months later. The NTX mice were prepared by thymectomy within 24 hours of birth with the use of published procedures [B. S. Mishell and S. M. Shiigi, *Selected Methods in Cellular Immunology* (Freeman, New York, 1980), pp. 326–331]. Techniques for isolating IELs used by our laboratory have been reported [R. L. Mosley and J. R. Klein, *J. Immunol. Methods* **156**, 19 (1992)]. IELs were isolated and studied from individual mice rather than as pooled preparations.
8. For analyses, we used fluorescein isothiocyanate (FITC)–labeled antibodies to mouse immunoglobulin (Zymed Laboratories, South San Francisco, CA); phycoerythrin (PE)–antibody to Thy-1.2 (anti–Thy-1.2) (CalTag, San Francisco, CA) and FITC– and PE–anti–Thy-1.1 (PharMingen); biotin–anti-TCRαβ (PharMingen); biotin–anti-TCRγδ (GL3) (PharMingen); streptavidin red 613 (Gibco BRL, Gaithersburg, MD); PE–anti-CD5 (PharMingen); PE–anti-CD4 (CalTag); FITC–anti-CD8α (CalTag); and PE-CD8β (PharMingen). For direct two-color staining, cells were reacted with a PE- and an FITC-labeled antibody, washed, fixed in 2% formaldehyde, and analyzed. For two-color TCR staining, cells were reacted with biotin-labeled anti-TCRαβ or anti-TCRγδ plus PE-labeled anti-CD5, washed, reacted with streptavidin red 613, washed, fixed, and analyzed on an Epics 751 flow cytometer interfaced to an MDADS II computer (Coulter, Hialeah, FL).
9. K. J. Maloy, A. M. Mowat, R. Zamoyska, I. N. Crispe, *Immunology* **72**, 555 (1991); L. Lefrancois, *J. Immunol.* **147**, 1746 (1991).
10. R. L. Mosley, J. Wang, M. Hamad, J. R. Klein, *Dev. Comp. Immunol.*, **18**, 155 (1994).
11. W. Pierpaoli and E. Sorkin, *Experimentia* **28**, 1385 (1972); W. Pierpaoli and H. O. Besedovsky, *Clin. Exp. Immunol.* **20**, 323 (1975).
12. W. Pierpaoli, H. G. Kopp, J. Muller, M. Keller, *Cell. Immunol.* **29**, 16 (1977); N. Farbis, *Int. J. Immunopathol. Pharmacol.* **5**, 93 (1992).
13. W. Pierpaoli and Y. Changxian, *J. Neuroimmunol.* **27**, 99 (1990).
14. K. Sakabe, K. Seiki, H. Fujii-Hanamoto, *Thymus* **8**, 97 (1986); B. L. Goff, J. A. Roth, L. H. Arp, G. S. Incefy, *Clin. Exp. Immunol.* **68**, 580 (1987); E. Ban *et al.*, *Life Sci.* **48**, 2141 (1990); M. Dardenne and W. Savino, *Ann. N.Y. Acad. Sci.* **650**, 85 (1992).
15. W. J. Murphy, S. K. Durum, D. L. Longo, *J. Immunol.* **149**, 3851 (1992); *J. Exp. Med.* **178**, 231 (1993).
16. Synthetic TRH peptide (*p*-Glu-His-Pro amide) (Sigma) treatment was patterned after that reported by others (*11*). This consisted of daily intraperitoneal injection of 100 μg of TRH for 5 days followed by six injections of 100 μg of TRH given every third day thereafter. Mice were tested 1 week or 3 weeks after initiation of TRH treatment.
17. Murine splenic and lymph node lymphocytes did not proliferate upon in vitro coculture with TRH either in the presence or in the absence of T cell–dependent cytokines under conditions used for mitogenic stimulation.
18. J. Wang and J. R. Klein, data not shown.
19. D. Kaiserlian, K. Vidal, J. O. Revillard, *Eur. J. Immunol.* **19**, 1513 (1989); K. Vidal, C. Samarut, J. P. Magaud, J. P. Revillard, D. Kaiserlian, *J. Immunol.* **151**, 4642 (1993).
20. J. R. Klein and M. F. Kagnoff, *J. Immunol.* **138**, 58 (1987); S. D. London *et al.*, *J. Exp. Med.* **165**, 830 (1987).
21. S. P. Balk *et al.*, *Science* **253**, 1411 (1991); D. Kaiserlian, K. Vidal, H. R. MacDonald, I. Grosjean, *Eur. J. Immunol.* **23**, 2717 (1993); P. Constant *et al.*, *Science* **264**, 267 (1994).
22. H. Schild *et al.*, *Cell* **76**, 29 (1994).
23. M. Holub, *Immunology of Nude Mice* (CRC Press, Boca Raton, FL, 1989), pp. 141–158; R. S. Henderson, B. McEvan, E. M. Pantelouris, *Thymus* **3**, 359 (1981); W. Pierpaoli and E. Sorkin, *Nature* **238**, 282 (1972); W. H. Weihe, *Exp. Cell Biol.* **52**, 140 (1984).

24. We thank M. Hamad, M. D. Howell, P. W. Kincade, K. S. Miller, R. L. Mosley, and B. S. Prabhakar for comments, discussions, and critical review of the manuscript. Supported by NIH grant DK35566 and by a grant from the Mervin Bovaird Center of the University of Tulsa.

1 June 1994; accepted 12 August 1994