variance matrices indicated strong sex linkage for genetic variation in age and size at maturity.

25. The *P* and *G* matrices were sampled from a multivariate normal distribution using the estimates of *P* and *G* as means and their sampling variance-covariance matrix as variance. The values for *R*, the vector of responses to selection, were sampled from a multivariate normal distribution with means equal to *R* (Table 1) and a sampling variance-covariance matrix obtained from the multivariate analysis of variance that compared the control and experimental populations. Vectors of 1000 values of β and *S* were calculated, and the distributions of β and *S* were inferred from these. Some of the sampled *G* matrices were not positive definite and hence could not be inverted. In these cases, the implied estimates of β and *S* were either positive or negative infinity, depending on the associated value of *R*, and were retained as such in our set of 1000 β and *S* vectors. If more than 25 of the 1000 estimates were positive infinity, the confidence interval was deemed to have no upper bound. Consequently, there are four different ways of reporting the results, depending on the nature of the *G* matrix and the results of the 1000 simulations: (i) One-sided confidence intervals are reported when singularity of more than 2.5% of the sampled *G* set the upper confidence limit at infinity. The one-sided value equals the 25th value of 1000 simulations (rank ordered from smallest to largest) and hence is equivalent to the lower bound of a 95% confidence interval. (ii) Confidence intervals with lower and upper bounds are reported when the simulations allowed us to set both an upper and lower limit to the distributions. The reported values are the 25th and 975th values of the 1000 simulations. (iii) Bivariate estimates of β and *S* were only possible for the males from both experiments. The only data set for which we could estimate the confidence limits for the bivariate analysis was the El Cedro males, for both the 4- and 7.5-year results. Here the limits mark the two-dimensional range of the 950 simulated values closest (in Euclidean distance) to the estimates. (iv) Standard errors were not estimable for the bivariate estimates of β and *S* for males from the Aripo River because of the near singularity of the *G* matrix. In this case, the difference between β values for age and size at maturity are more pronounced in the bivariate analysis, which takes the high genetic correlation between them into account, than in the univariate analysis.

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Local Hormone Networks and Intestinal T Cell Homeostasis

Jin Wang, Michael Whetsell, John R. Klein*

Neuroendocrine hormones of the hypothalamus-pituitary-thyroid axis can exert positive or negative immunoregulatory effects on intestinal lymphocytes. Small intestine epithelial cells were found to express receptors for thyrotropin-releasing hormone (TRH) and to be a primary source of intestine-derived thyroid-stimulating hormone (TSH). The gene for the TSH receptor (TSH-R) was expressed in intestinal T cells but not in epithelial cells, which suggested a hormone-mediated link between lymphoid and nonhematopoietic components of the intestine. Because mice with congenitally mutant TSH-R (*hyt/hyt* mice) have a selectively impaired intestinal T cell repertoire, TSH may be a key immunoregulatory mediator in the intestine.

The intestine constitutes an important host barrier to foreign antigen entry. This is reflected in the extensive complexity of the intestinal immune system, which is characterized by novel lymphocyte subsets (*1*) and by bidirectional intercellular communication between lymphocytes and epithelial cells (*2*). We recently demonstrated a role for neuroendocrine hormones in the development and regulation of intestinal T cells, in particular the TCRαβ, CD8αβ intraepithelial lymphocytes (IELs) (*3*, *4*). Here, we describe a pathway of hormone synthesis and use mediated by thyrotropin (TSH), which links components within the small intestine and is used in local IEL immune regulation.

Freshly extracted small intestine cells (*5*) were characterized by flow cytometric analysis (*6*, *7*). Populations of epithelial cells and lymphocytes were enriched to >97% purity, as verified by reactivity with monoclonal antibody (mAb) G8.8, a marker of murine epithelial cells, and a mAb to the CD45 leukocyte-common antigen (LCA), a marker of nucleated hematopoietic cells (*8*–*10*) (Fig. 1). Purified IELs and epithelial cells were assayed for expression of the TSHβ gene by reverse transcriptase–polymerase chain reaction (RT-PCR) (*11*). This yielded PCR products of the predicted size from both the IEL-enriched fraction and the epithelial cell–enriched fraction (Fig. 2), which were confirmed (*12*) by DNA sequence analyses (*11*). Because TSH production is controlled in part by TRH, purified IELs and epithelial cells were assayed for TRH receptor (TRH-R) gene expression by RT-PCR (*11*). A PCR product of the anticipated size was obtained from intestinal epithelial cells, whereas no PCR product was obtained from intestinal IELs (Fig. 2). The PCR product identified in epithelial cells was verified by reamplification using a nested upstream TRH-R primer located within the amplification region (*11*); this resulted in a single band of the anticipated 146–base pair size with sequence homology to murine TRH-R (*11*, *13*).

Secretion of TSH by IELs and epithelial cells was measured by enzyme-linked immunosorbent assay (ELISA) (*14*) using supernatants of cells cultured with and without TRH according to published protocols (*15*). Although TSHβ was produced by both IELs and epithelial cells, epithelial cells produced considerably more TSHβ than did an equivalent number of IELs (Fig. 3A), with maximal secretion occurring at 10^{-7} to 10^{-9} M TRH. This secretion pattern, including the high-dose prozone effect of TRH, is similar to previous reports of TRH-induced TSH secretion (*15*). TSH was detected in epithelial cell supernatants as early as 1 hour after stimulation (Fig. 3B), implying

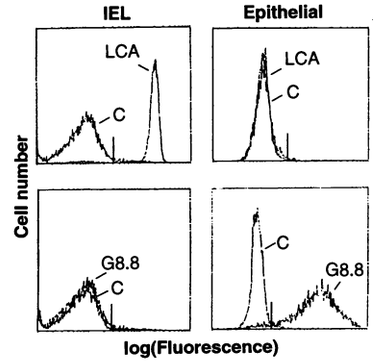

Fig. 1. One-color flow cytometric analyses, showing reactivities of LCA and epithelial cell antigen (G8.8) mAbs for IEL and epithelial cell populations. C, isotype-species–matched control mAb.

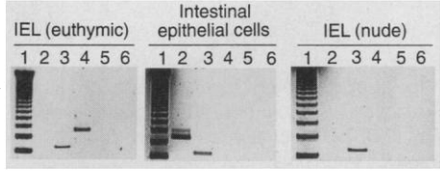

Fig. 2. RT-PCR analyses of gene expression in intestinal IELs and intestinal epithelial cells from euthymic mice and intestinal IELs from congenitally athymic nude mice. Lane 1 (in each panel), base pair standards; lanes 2 to 4, RT-PCR–amplified gene products for TRH-R, TSHβ, and TSH-R, respectively; lane 5, primer controls in the absence of cDNA templates; and lane 6, controls for DNA contamination of RNA (that is, PCR analyses of RNA preparations after treatment with deoxyribonuclease but before cDNA construction).

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. E-mail: john-klein@centum.utulsa.edu

that TSH produced by those cells is probably regulated at the posttranscriptional stage from preexisting transcripts. Unlike epithelial cells, there was a slight but not statistically significant ($P > 0.05$) increase in TSH production by IELs in response to TRH (Fig. 3B), which could reflect cross-reactivity of TRH on receptors other than TRH-R (*16*). Although some epithelial cells and IELs appear to produce TSH constitutively (Fig. 3, A and B), those cells also may have been activated in situ by TRH or perhaps by endotoxin (*16, 17*).

To determine which cells in the small intestine were responsive to TSH, we studied the expression of the TSH receptor (TSH-R) gene (*11, 14*) in purified preparations of IELs and epithelial cells. A PCR product with sequence homology to the murine TSH-R gene (*14*) was obtained from intestinal IELs (Fig. 2) (*11*); no PCR product was obtained from intestinal epithelial cells. Because previous studies have demonstrated that IELs from congenitally athymic nude mice do not respond to exogenous TSH therapy (*3*), TRH-R, TSHβ, and TSH-R gene expression was studied in IELs from nude mice. Similar to euthymic mice, IELs from nude mice expressed the TSHβ gene and did not express the TRH-R gene (Fig. 2); however, they failed to express the TSH-R gene, which explains why IELs in these animals fail to respond to TRH-TSH therapy (*3*). The experiments described above were reproducible in three euthymic and two athymic BALB/c or C57BL/6 mice, and the RT-PCR pattern obtained for epithelial cells was observed using the MODE-K murine small intestine cell line (*18*).

To study the surface expression of TRH and TSH receptors on IELs and epithelial cells as well as on lymphoid cells outside the intestine, we used ^{125}I-labeled TRH and TSH binding assays (*19–21*). TRH did not bind appreciably to T cells from the spleen, peripheral lymph nodes, or IELs; however, compared to those cells, there was a >15-fold increase in TRH binding to freshly extracted small intestine epithelial cells and MODE-K cells (Fig. 3C) (*18*). No differences were noted in the pattern of TRH binding to nude mouse IELs or epithelial cells relative to that seen in euthymic mice. In contrast, binding of ^{125}I-labeled TSH to T cells from IELs was ~50 times the amount of binding to intestine epithelial cells or MODE-K cells and was significantly greater ($P < 0.01$) than TSH binding to T cells from the spleen or peripheral lymph nodes (Fig. 3D). Unlike euthymic mice, IELs from nude mice did not bind TSH. These findings demonstrate a differential pattern of hormone use by distinct cell populations in the small intestine and identify a hormone defect associated with IELs in congenitally athymic mice.

To explore the extent to which TSH is functionally involved in IEL immunoregulation, we studied T cell populations in *hyt/hyt* mice, which have a congenital point mutation in the TSH-R gene resulting in a Pro→Leu substitution in the TSH-R polypeptide (*22, 23*). Because *hyt/hyt* mice have elevated levels of biologically active serum TSH but are unable to use TSH (*22, 23*), these mice permit a comparison between the IELs in animals with minimal capacity to use TSH and those of mice that use TSH normally. Relative to normal mice, *hyt/hyt* mice had one-third to one-fourth as many total IELs, a slight decrease in CD8αα$^+$ and TCRγδ$^+$ IELs, and only 3.3 to 4% as many TCRαβ$^+$ and CD8αβ$^+$ IELs ($P < 0.001$), as calculated from the proportion of cell staining by flow cytometry and the total number of cells recovered per mouse (Fig. 4A). These changes in *hyt/hyt* mice were similar to the hormone-associated IEL perturbations in neonatally thymectomized mice (*3*); in both, CD4$^+$8$^-$ IELs were largely unaffected. The numbers of CD4$^-$8$^-$ IELs in *hyt/hyt* mice were the same as in normal mice (Fig. 4A), suggesting that the TSH-related developmental defect of *hyt/hyt* mice restricts the ability of some immature CD4$^-$8$^-$ IELs (*24*) to proceed to maturity. Outside the intestine, T cell numbers in *hyt/hyt* mice were about half those of normal mice, as shown in Fig. 4B for spleen cells. CD8$^+$ T cells in the spleen were exclusively CD8αβ$^+$, indicating that the reduction in CD8αβ IELs in *hyt/hyt* mice was not the result of a defect in CD8 β-chain expression. These patterns held true for T cells in the peripheral lymph nodes, Peyer's patches, and thymus of *hyt/hyt* mice tested between 8 and 20 weeks of age, and were independent of environmental factors associated with animal housing (*5*).

The paracrine-autocrine network described here explains how intestinal T cells can be regulated locally by hormones, because it places the source, induction, and use of TSH within the intestine itself. Although lymphoid cells outside the intestine are capable of binding TSH (Fig. 3) (*25*), the finding of greater TSH binding to IELs than to extra-intestinal T cells suggests that differences exist in the use of TSH between those populations, which also may involve variations in hormone-mediated signaling. The restricted ability of both *hyt/hyt* and nude mice to use TSH, in combination with the differences in IELs between these mice

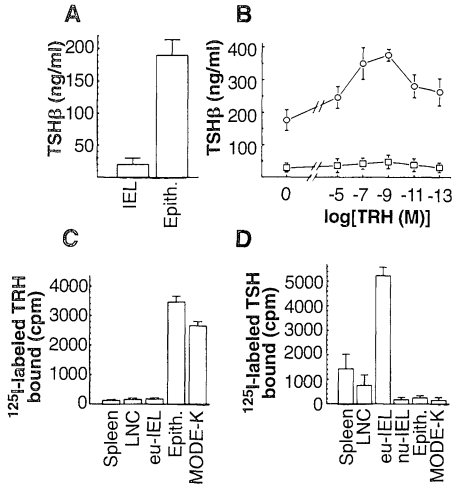

Fig. 3. (**A**) TSH secretion by IELs and epithelial cells after 48 hours of culture in the absence of TRH. (**B**) TSH secretion by IELs (□) and epithelial cells (○) in the presence of varying concentrations of TRH; values are means ± SEM of three experiments assayed at 1, 6, and 48 hours after stimulation. (**C** and **D**) Relative binding of ^{125}I-labeled TRH (C) and ^{125}I-labeled TSH (D) to splenic T cells, peripheral lymph node T cells (LNC), euthymic (eu) and nude (nu) mouse IELs, intestinal epithelial cells, and the MODE-K murine small intestine cell line. Values are means plus the range of radioactive hormone binding of triplicate cultures from four or five mice. Statistical analyses were done by Student's *t* test for unpaired observations.

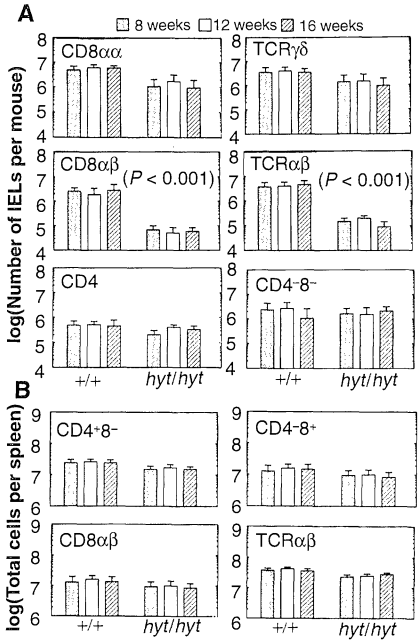

Fig. 4. (**A**) Numbers of CD8αα$^+$, CD8αβ$^+$, CD4$^+$, CD4$^-$8$^-$, TCRαβ$^+$, and TCRγδ$^+$ IELs per small intestine, and (**B**) numbers of CD4$^+$8$^-$, CD4$^-$8$^+$, CD8αβ$^+$, and TCRαβ$^+$ cells per spleen, of +/+ and *hyt/hyt* mice at 8, 12, and 16 weeks of age calculated from flow cytometric analyses and total numbers of cells isolated. *P* values indicate statistically significant differences in the numbers of cells in *hyt/hyt* mice relative to normal mice at each time point as determined by Student's *t* test for unpaired observations. Values are means plus the range of numbers of cells obtained from three to seven +/+ or *hyt/hyt* mice analyzed separately.

and normal mice, suggests that TSH is an essential component of IEL homeostasis. Although our studies do not exclude the possibility that TSH activates secondary hormone responses such as thyroid hormones or corticosteroids, this is unlikely because *hyt/hyt* mice have normal levels of corticosteroids (*26*) and because we found no increases in the numbers of TCRαβ or CD8αβ IELs in *hyt/hyt* mice supplemented with thyroxine for 3 weeks starting at 3 or 6 weeks of age (*4*, *26*). Given that TSH can be stored in secretory granules (*27*, *28*), is released from epithelial cells shortly after TRH stimulation (Fig. 3B), and has a short half-life in vivo (*28*), a TSH-mediated signal used for IEL immunoregulation would occur rapidly. A dynamic interactive hormone system such as this could efficiently adjust the distribution of IELs in selected regions of the intestine under normal conditions and may explain the localized nature of intestinal immunopathologies in various disease states.

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11. RNA extracted from IELs and epithelial cells was treated with deoxyribonuclease (BRL, Gaithersburg, MD) for 15 min at 25°C and converted into cDNA using reverse transcriptase (BRL) according to procedures previously reported by this laboratory (*29*). Primers were constructed from published sequences for TSHβ, TRH-R, and TSH-R, respectively (*12*, *13*, *23*): TSHβ upstream, 5′-GAGTGTGGCTACTGCCTGACC-3′; TSHβ downstream, 5′-ACACTTGCCACACTTG-CAGCT-3′; TRH-R upstream, 5′-ATGTTGTGCCAAT-GATCCTG-3′; TRH-R downstream, 5′-TAGGGCCA-CACTGTAGTTAGC-3′; TSH- R upstream, 5′-GCG-TCTCCACCCTGTGAGTGTCA- CC-3′; and TSH-R downstream, 5′-CATGTAAGGGTTGTCTGTGATTTC-3′. Amplification was done using a 40-cycle program consisting of 94°C for 45 s, 65°C for 45 s, and 72°C for 15 s. For DNA sequencing, the PCR-amplified products shown in Fig. 2 were diluted 1:5000 and reamplified using a nested upstream primer and the original downstream primer (*11*). DNA sequencing was done using fluorescent dye–labeled dideoxynucleotides with the nested primers with Taq polymerase. Products were analyzed on a Biosystems 370A automated DNA sequencer (Applied Biosystems). Nested upstream primers were as follows: TRH-R, 5′-AGGGCTG-GAGAGAAATGAGTTGAC-3′; TSH-R, 5′-GACT-CATCTGAAGACCATACCCAGTCTTGCA-3′; and TSHβ, 5′-CTGTTTCTTCCCAAATATGCACTC-3′. Sequences obtained from the PCR products using the primers described above were identical to published sequence regions for the appropriate genes: TSHβ, 291–413 (*12*); TRH-R, 970–1115 (*13*); and TSH-R, 215–491 (*23*).
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