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Developmental control of lymphokine gene expression in fetal thymocytes during T-cell ontogeny

(growth factors/*in situ* RNA hybridization)

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ABSTRACT **We have used the technique of *in situ* hybridization to investigate the expression of lymphokine genes by immature thymocytes during intrathymic development. In 13-day fetal thymocytes a population of cells constitutively produces low levels of interleukin 2 (IL-2) and interleukin 4 (IL-4) mRNAs. A second phase of lymphokine gene expression occurs in the majority of 15-day thymocytes, and a population of cells constitutively produces both IL-2 and IL-4 mRNAs. Thymocytes at 14 days of gestation and after 16 days up until birth do not express detectable lymphokine mRNA. By contrast, the population of IL-2 receptor mRNA-producing thymocytes increases progressively up to 15 days of gestation, and expression thereafter decreases up to birth. In addition, thymocytes expressing interferon γ mRNA were not present until just prior to birth. Our findings indicate developmental control of lymphokine and lymphokine receptor gene expression in fetal thymocytes during ontogeny.**

A central issue of T-cell ontogeny is the identification of the signals and growth factors required for the proliferation and differentiation of immature cells in the thymus. Studies seeking to examine this issue by measuring the production and use of growth factors by thymocytes have depended on the stimulation of these cells by phorbol esters and ionophores outside the thymic environment (1–5). These results may not be representative of events inside the intact organ for several reasons. First, the activating agents are not physiological, and second, cells stimulated in this way do not undergo normal maturation or full T-cell receptor gene rearrangement. In addition, the development of thymocytes within the thymus may in part be dependent upon the coordinated production of growth factors by different populations of cells throughout ontogeny. Thus, the use of such potent stimulating agents as phorbol esters and ionophores to induce lymphokine production by immature thymocytes *in vitro* may mask or abrogate the regulated production of these growth factors by thymocytes that would occur *in vivo*.

To establish a role for any gene in thymic development, it is essential that its expression by immature thymocytes be demonstrated during intrathymic development. In this study, therefore, the expression of the genes for the growth factors interleukin 2 (IL-2) and interleukin 4 (IL-4) for IL-2 receptors and for interferon γ (IFN-γ) has been assessed in thymocytes freshly isolated from carefully staged mouse embryos. The results clearly show that during defined periods of ontogenesis *in vivo* differentiating thymocytes produce mRNA for both IL-2 and IL-4 as well as for the IL-2 receptors. By contrast, IFN-γ production is not evident until just prior to birth.

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MATERIAL AND METHODS

Hybridization Probes. All probes used for *in situ* hybridization were cDNA fragments inserted into the polylinker site of the pGEM-3 or -4 plasmids (Promega) by using standard procedures. The IL-2 probe, kindly provided by V. Paetkau (University of Alberta, Edmonton) was a 900-base-pair (bp) *Pst* I–*Eco*RI fragment corresponding to a full-length cDNA clone (6). The IL-2 receptor probe, generously provided by S. Gillis (Immunex, Seattle), consisted of a full-length cDNA clone subcloned in an SP64 plasmid (7). The IL-4 probe consisted of a 236-bp *Pst* I–*Rsa* I fragment derived from a cDNA clone kindly provided by T. Honjo (Kyoto University, Kyoto, Japan) (8). The IFN-γ probe was prepared by subcloning of a 641-bp *Pst* I–*Rsa* I fragment isolated from a full-length cDNA clone kindly provided by F. Lee (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) (9). After linearization of the pGEM plasmid DNA containing the appropriate insert, RNA sense or anti-sense transcripts were synthesized by using SP6 or T7 RNA polymerase according to the supplier's recommendations (Promega).

***In Situ* Hybridization.** CBA/Ca embryonic thymus lobes were dissected from fetal mice of known gestational state (date of detection of a vaginal plug was counted as day 0). The lobes were gently dispersed to yield thymocyte suspensions. *In situ* hybridization was carried out on cytocentrifuge preparations of embryonic thymocytes essentially as described previously (10, 11).

RESULTS AND DISCUSSION

As shown in Fig. 1 and Table 1, fetal thymocytes [freshly isolated from thymic lobes of 13- to 18-day embryos and newborns (20 days)] that constitutively produce IL-2, IL-4, and IL-2 receptor mRNA can be detected. The population of IL-2 receptor mRNA-producing thymocytes is much larger than the population(s) producing IL-2 and IL-4 mRNAs and appears to increase progressively up to 15 days of gestation in parallel with the levels of cell surface IL-2 receptors detected by several groups using anti-IL-2 receptor antibodies (12–15). By contrast, there is a striking temporal distribution of thymocytes producing both IL-2 and IL-4 mRNAs. Among 13-day thymocytes a significant number of cells constitutively produce IL-2 mRNA and a much smaller number produce IL-4 mRNA. A second phase of lymphokine production occurs in 15-day thymocytes, in which the frequency of IL-2- and IL-4-producing cells is much higher. In between these two waves of lymphokine production, 14-day

Abbreviations: IL-2, interleukin 2; IL-4, interleukin 4; IFN-γ, interferon γ.

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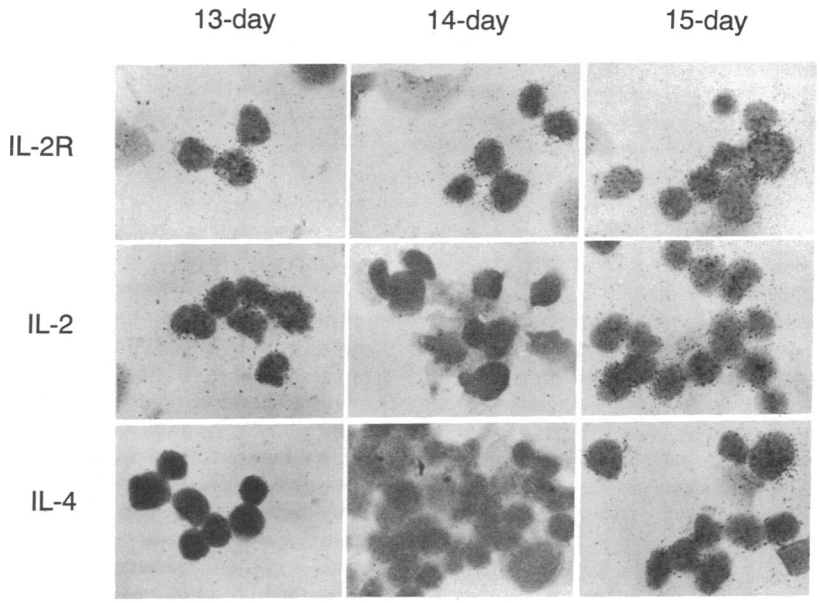

FIG. 1. Detection of constitutive lymphokine and lymphokine receptor mRNA production in fetal thymocytes by *in situ* hybridization. Fetal thymocytes were obtained from CBA fetal mouse thymic rudiments after timed matings with the day of detection of a vaginal plug designated day 0. Cytocentrifuge preparations of thymocytes were hybridized with an ^{35}S-labeled RNA probe complementary to IL-4, IL-2, or IL-2 receptor (IL-2R) RNA, synthesized by *in vitro* transcription from cDNA templates inserted into pGEM vectors by using either SP6 or T7 RNA polymerase. After 25 days of autoradiography the sides were developed and counterstained, and representative fields from three independent experiments were photographed. (×700.)

fetal thymocytes do not express any detectable IL-2 or IL-4 mRNA. Similarly, 18- and 20-day fetal thymocytes also do not show any IL-2 or IL-4 gene expression. The specificity of hybridization was confirmed by using IL-2, IL-4, and IL-2 receptor probes of the sense orientation (i.e., same polarity as cellular RNA). No detectable hybridization of any of these probes was seen in any preparation of thymocytes (Fig. 2 *a* and *b*). This dramatic temporal distribution of thymocytes producing IL-2 and IL-4 mRNA was consistently reproduced in four independent experiments.

As shown in Table 1, the expression of IL-2 mRNA, determined by the number of grains per cell, is low in 13-day fetal thymocytes. An almost 2-fold increase in IL-2 mRNA is, however, seen in 15-day thymocytes. Likewise, the IL-4 mRNA-producing thymocytes detected at 13 days of gestation are present at low levels although a 2-fold increase is seen in IL-4 mRNA produced by 15-day thymocytes. By 16 days of gestation, the number of thymocytes expressing IL-2 and IL-4 mRNAs decreases dramatically: approximately 1 in 10 cells expresses IL-2 mRNA and 1 in 30 produces IL-4 mRNA. By 18 days of gestation, IL-2 and IL-4 mRNAs cannot be detected. The low levels of lymphokine mRNA in 13- and 15-day thymocytes may explain why it has previously not been possible to detect constitutive IL-2 and/or IL-4 mRNA production by fetal thymocytes with conventional Northern blot analysis. This point is further emphasized by comparing the levels of IL-2 mRNA in fetal thymocytes (Fig. 1) and in individual mitogen-stimulated normal CD4$^+$ T cells shown in Fig. 3. The average grain count per CD4$^+$ T cell, 24 hr after concanavalin A activation (data not shown), is 3 to 4 times the level of IL-2 mRNA in 15-day fetal thymocytes. Although the levels of IL-4 and IL-2 mRNA produced by individual 15-day thymocytes are low, the frequency of IL-2- (87%) and IL-4- (45%) mRNA-expressing thymocytes is high, indicating that some populations of thymocytes produce both lymphokines. This is in contrast to previous data from our own (17) and other (18) laboratories, which has shown by using monoclonal T-cell lines that it is possible to identify distinct subsets of mature CD4$^+$ T cells on the basis of the production of either IL-2 or IL-4 after activation with antigen or mitogen.

These observations suggest that lymphokine production by immature thymocytes is tightly controlled during their intrathymic development and that they may be receptive to the inductive signals required for lymphokine production only at distinct stages of their development *in vivo*. These findings may provide an explanation for the recent observations of Sideras *et al.* (4) that the ability of fetal thymocytes to produce IL-4 mRNA after stimulation *in vitro* appears to be dependent upon the gestational age of the fetal thymocytes. In contrast with the present study, however, no attempt was made to investigate the production of lymphokines *in vivo*

Table 1. Constitutive lymphokine and lymphokine receptor gene expression by thymocytes during embryonic development

mRNA detected	% cells positive at gestational age						Grains per cell					
	13 days	14 days	15 days	16 days	18 days	20 days	13 days	14 days	15 days	16 days	18 days	20 days
IL-2	17	2	87	11	<1	<1	12 ± 4	2 ± 1	24 ± 6	9 ± 3	4 ± 2	2 ± 1
IL-4	5	1	45	3	<1	<1	8 ± 2	2 ± 1	17 ± 4	7 ± 3	3 ± 1	2 ± 1
IFN-γ	<1	<1	2	<1	3	5	2 ± 1	2 ± 1	4 ± 2	4 ± 1	7 ± 3	9 ± 2
IL-2 receptor	38	95	95	35	18	9	10 ± 4	27 ± 9	21 ± 4	12 ± 4	8 ± 4	6 ± 2

Cytocentrifuge preparations of thymocytes obtained from embryos of different gestational ages were analyzed for lymphokine gene expression by *in situ* hybridization using complementary ^{35}S-labeled IL-2, IL-4, IL-2 receptor, and IFN-γ RNA probes. Cells with more than 5 grains per cell were counted as positive. More than 500 cells from 10 different microscope fields of slides from several independent experiments were counted to obtain the mean grain count per cell, which is given ±SD.

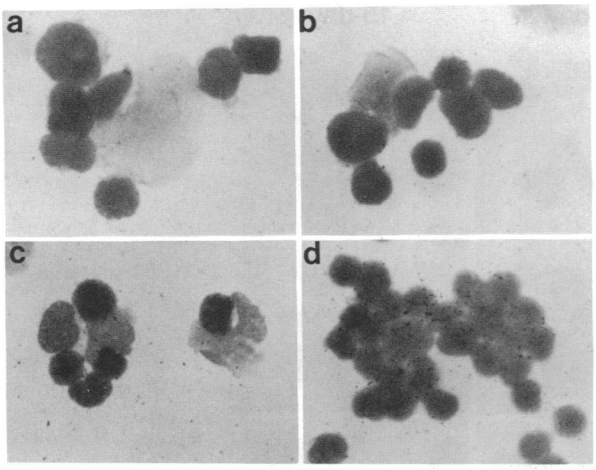

FIG. 2. *In situ* hybridization of IL-2 and IL-4 sense RNA probes and a complementary IFN-γ RNA probe with fetal thymocytes. Thymocytes from 15-day embryonic CBA mice were hybridized with ^{35}S-labeled IL-2 (*a*) or IL-4 (*b*) RNA probes transcribed in the sense orientation. Thymocytes from 15-day fetal (*c*) or 20-day, newborn (*d*), CBA mice were also hybridized with a complementary ^{35}S-labeled IFN-γ RNA probe. (×550.)

during thymic development. The virtual absence of any IFN-γ mRNA-producing fetal thymocytes (Fig. 2*c*, Table 1) is in contrast to the findings from a recent study demonstrating that 15-day fetal thymocytes produce IFN-γ after activation *in vitro* with phorbol ester and ionophore (30). However, as the physiological equivalents of the activation signal(s) provided by these agents are not known, it is difficult to interpret these findings as representing the production of IFN-γ by fetal thymocytes *in vivo*. Interestingly, a subset of 18-day and 20-day (newborn) thymocytes, which at that stage consist of populations of more mature thymocytes of the helper and cytolytic phenotype (19, 20), constitutively express low levels of IFN-γ mRNA (Fig. 2*d*, Table 1).

The findings that both the highest frequency of IL-2 receptor mRNA-expressing thymocytes and the highest levels of IL-2 receptor mRNA produced per cell closely parallel those thymocytes producing IL-2 mRNA suggest that both may be coordinately expressed to promote growth and differentiation of populations of T-cell precursors. The frequency of IL-2 receptor mRNA-producing thymocytes, as shown here, correlates well with the number of thymocytes expressing cell surface IL-2 receptors (12–15) during intrathymic development. IL-2 receptor expression can be measured only by antibody to the nonfunctional IL-2 receptor chain, so the results cannot be definitive with regard to IL-2 usage. However, the observation that the addition of exogenous IL-2 can reverse the inhibition by IL-2 receptor antibodies of growth and differentiation of thymocytes in thymic organ cultures (21) suggests that the IL-2 receptor mRNA detected encodes functional receptors in at least some populations of fetal thymocytes. In addition, evidence has been obtained that cell surface IL-2 receptor-positive thymocytes expressing cytoplasmic T-cell receptor β chains are mitotic cells, whereas IL-2 receptor-negative cells that express surface β chains are cycling slowly or not at all (22). The apparent failure to demonstrate fetal thymocyte proliferation in response to exogenously added IL-2 in previous studies (12, 23) may be indicative of a requirement for additional lymphokines distinct from IL-2 (for example, IL-4), which coordinately act to promote thymocyte growth and expansion. Indeed, the observation that IL-2 receptor-bearing CD4$^-$, CD8$^-$ thymocytes are able to proliferate in response to IL-2 in the presence of concanavalin A (24) suggests that additional lymphokines, induced in this case by mitogen, in combination with IL-2 may provide the necessary signals for the proliferation of T-cell precursors.

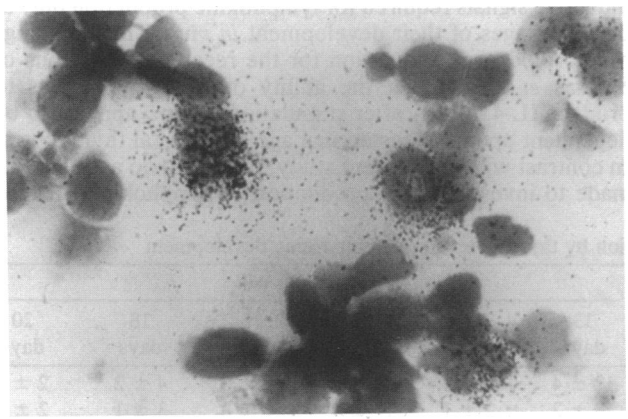

FIG. 3. IL-2 mRNA production in mitogen-stimulated CD4$^+$ T cells detected by *in situ* hybridization. CD4$^+$ T cells isolated from total spleen cells (16) of CBA mice were cultured for 24 hr in the presence of concanavalin A (2.5 μg/ml) and hybridized *in situ* with a complementary ^{35}S-labeled IL-2 RNA probe. Autoradiography was carried out for 12 days. (×550.)

Our results provide direct evidence that maturing fetal thymocytes freshly derived from the embryonic thymus produce IL-2 and IL-4 mRNA. These studies suggest that different populations of fetal thymocytes may utilize distinct growth factors or, alternatively, that thymocytes produce and respond to different lymphokines at different stages of their intrathymic development. Although these growth factors may be crucial for proliferation of fetal thymocytes from the 13th day of gestation, other distinct signals may be required for the proliferation of fetal thymocytes present in the 12-day thymus and for rearrangement of T-cell receptor genes. For example, evidence has been obtained for the involvement of CD3$^+$ cells in the fetal thymus in the rearrangement of the β T-cell receptor genes (25).

The signals inducing IL-2 and IL-4 gene expression in thymocytes within the thymus are presently unknown. A recent study demonstrating that 13-day fetal thymocytes activated *in vitro* with anti-Thy.1 or anti-CD3 antibodies produce IL-2 and IL-4 (25) suggests that Thy.1 or CD3 molecules may act as signal transducers for the induction of lymphokine production in thymocytes during ontogenesis. However, the observations (*i*) that the number of 13-day fetal thymocytes which express IL-2 or IL-4 mRNA *in vivo*, as shown in this study, is much less than the number of 13-day thymocytes expressing Thy.1 (26) and (*ii*) that it is not clear as to whether cell surface CD3 can be functionally expressed in 13-day fetal thymocytes (27, 28) make it unlikely that activation of 13-day fetal thymocytes through cell surface Thy.1 or CD3 molecules can fully account for the production of IL-2 and IL-4 by these cells as seen in this study.

In view of the recent demonstration of IL-4 receptors on the surface of fibroblasts and epithelial cells, which in the presence of IL-4 are induced to produce colony-stimulating activity (29), it is possible that soluble factors produced by either thymic stromal elements or thymocytes themselves provide the signals for the production of growth factors by either cell type mediating proliferation and maturation of thymocytes in the thymus.

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1. Kisielow, P., Leiserson, W. & von Boehmer, H. (1984) *J. Immunol.* **133,** 1117–1123.
2. Hardt, C., Diamantstein, T. & Wagner, H. (1985) *J. Immunol.* **134,** 3891–3894.
3. Zlotnik, A., Ransom, J., Frank, G., Fischer, M. & Howard, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3856–3860.
4. Sideras, P., Funa, K., Zalcberg-Quintana, I., Xanthopoulos, K., Kisielow, P. & Palacios, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 218–221.
5. Palacios, R., Sideras, P. & von Boehmer, H. (1987) *EMBO J.* **6,** 91–95.
6. Kashima, N., Nishi-Takaoka, C., Fujita, T., Taki, S., Yamada, G., Hamuro, J. & Taniguchi, T. (1985) *Nature (London)* **313,** 402–404.
7. Cosman, D., Wignall, J., Lewis, A., Alpert, A., McKereghan, K., Cerretti, D. P., Gillis, S., Dower, S. & Urdal, D. (1987) in *Lymphokines: Molecular Cloning and Analysis of Lymphokines,* eds. Webb, D. R. & Goeddel, D. V. (Academic, New York), Vol. 13, pp. 109–121.
8. Noma, Y., Sideras, P., Naito, T., Bergstedt-Lindquist, S., Azuma, C., Severinson, E., Tanabe, T., Kinashi, T., Matsuda, F., Yaoita, Y. & Honjo, T. (1986) *Nature (London)* **319,** 640–646.
9. Gray, P. W. & Goeddel, D. V. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5842–5846.
10. Harper, M. E., Marselle, L. M., Gallo, R. C. & Wong-Staal, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 772–776.
11. Jones, B., Carding, S. R., Kyes, S., Mjolsness, S., Janeway, C., Jr., & Hayday, A. (1988) *Eur. J. Immunol.* **18,** 1907–1915.
12. von Boehmer, H., Cristani, A., Kisielow, P. & Haas, W. (1985) *Nature (London)* **314,** 539–540.
13. Habu, S., Okumura, K., Diamantstein, T. & Shevach, E. M. (1985) *Eur. J. Immunol.* **15,** 456–460.
14. Owen, M. J., Jenkinson, E. J., Brown, M. H., Sewel, W. A., Krissansen, G. W., Crumpton, M. J. & Owen, J. J. T. (1988) *Eur. J. Immunol.* **18,** 187–189.
15. Takacs, L., Osawa, H. & Diamantstein, T. (1984) *Eur. J. Immunol.* **14,** 1152–1156.
16. Wigzell, H. (1976) in *In Vitro Methods in Cell Mediated and Tumor Immunity*, eds. Block, B. R. & David, J. R. (Academic, New York), pp. 245–249.
17. Killar, L., MacDonald, G., Wood, A., West, J. & Bottomly, K. (1987) *J. Immunol.* **138,** 1674–1679.
18. Mosmann, T., Cherwinski, R. H., Bond, M. W., Griedlin, M. A. & Coffman, R. L. (1986) *J. Immunol.* **136,** 2348–2357.
19. Ceredig, R., Dialynas, D. P., Fitch, F. W. & MacDonald, H. R. (1983) *J. Exp. Med.* **157,** 1654–1671.
20. Ceredig, R., MacDonald, H. & Jenkinson, E. J. (1983) *Eur. J. Immunol.* **13,** 185–190.
21. Jenkinson, E. J., Kingston, R. & Owen, J. J. T. (1987) *Nature (London)* **329,** 160–162.
22. Parkin, I. G., Owen, J. J. T. & Jenkinson, E. (1988) *Immunology* **64,** 97–99.
23. Lugo, J. P., Krishnan, S. N., Sailor, R. D., Koen, P., Malek, T. & Rothenberg, E. (1985) *J. Exp. Med.* **161,** 1048–1056.
24. Raulet, D. H. (1985) *Nature (London)* **314,** 101–103.
25. Owen, J. J. T., Owen, M. J., Williams, G. T., Kingston, R. & Jenkinson, E. (1988) *Immunology* **63,** 639–642.
26. Pardoll, D. M., Fowlkes, B. J., Lechler, R. I., Germain, R. N. & Schwartz, R. H. (1987) *J. Exp. Med.* **165,** 1624–1638.
27. Tentori, L., Pardoll, D. M., Zuniga, J. C., Hu-Li, J., Paul, W. E., Bluestone, J. A. & Kruisbeek, A. M. (1988) *J. Immunol.* **140,** 1089–1094.
28. Bluestone, J. A., Pardoll, D., Sharrow, S. O. & Fowlkes, B. J. (1987) *Nature (London)* **326,** 82–84.
29. Park, L. S., Tushinski, R. J., Mochizuki, D. Y. & Urdal, D. L. (1988) *J. Cell. Biochem. Suppl.* **12A,** 111.
30. Ransom, J., Fischer, M., Mosmann, T., Yokota, T., DeLuca, D., Schumacher, J. & Zlotnik, A. (1987) *J. Immunol.* **139,** 4102–4108.