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Analysis by *in situ* hybridization of cytokine mRNA expression in the murine developing thymus

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Abstract

We have used *in situ* hybridization to investigate the expression of IL-1, IL-2, IL-4, IL-6 and IFN-γ genes by thymic cells during fetal development in mice. Two waves of mRNAs were detected in thymic cells for IL-1 at days 16 and 19 of gestation, for IL-2 at days 14 and 18, and for IL-4 at days 14 and 16. Three peaks for IL-6 were observed at days 13, 17 and around birth. Finally, only one peak of cells positive for IFN-γ was detected. Whereas cells positive for IL-1 were generally grouped and more often localized in the external area of the thymus, the other positive cells were isolated and evenly distributed in the thymus. Our results illustrated the presence of cytokine transcripts in the developing thymus following a developmentally controlled sequence and support the hypothesis that cytokines could play a role in T cell development.

Introduction

The thymus is the central lymphoid organ responsible for the production of immunocompetent T cells. These lymphocytes differentiate from precursors deriving from fetal liver and from hematopoeitic marrow (1) which generate functionally competent T cells after several stages of cellular proliferation and differentiation related to sequential interactions with several types of thymic stromal cell (2–4).

The role of cytokines in T cell development has been suspected from *in vitro* observations: thymocytes (5–7) and thymic epithelial cells (8–11) have been shown to produce cytokines and to respond to their actions (5,12–18).

The cytokines most likely to be involved in T cell development are those produced by thymic cells under physiological conditions. However, until now, most of the data concerning this production were obtained *in vitro*, often after stimulation with non-physiological agents such as phorbol myristate acetate or calcium ionophores. The first demonstration of the presence of cytokines in the thymus was the detection of IL-2 and IL-4 transcripts in suspensions of non-stimulated thymocytes isolated from fetuses (19). Later, we demonstrated that mRNAs for tumor necrosis factor (TNF)-α were present on frozen thymus sections at day 13 of gestation and around birth (day 19).

In this work, we extended these *in situ* observations and used RNA–RNA *in situ* hybridization on frozen thymic sections to study the presence of cells expressing IL-1, IL-2, IL-4, IL-6 and IFN-γ transcripts in the developing thymus of mice.

Methods

Mice

C57BL/KA mice originating from Stanford University were raised in our animal colony. Mice were mated for 1 night and the fetuses were removed at various days of gestation (date of detection of a vaginal plug was counted as day 0).

Tissue and cytocentrifuge preparations

The embryonic lobes were gently dispersed to yield thymocyte suspensions used to prepare cytocentrifuge preparations or were frozen in liquid nitrogen. Cryosections of frozen thymuses (5–6 μm) prepared at –20°C were placed on glass slides coated with 0.1 mg/ml of poly-L-lysine (Sigma, Deisenhofen, Germany). For *in situ* hybridization, the sections or the cytocentrifuge preparations were fixed in paraformaldehyde (4% in PBS) containing 20 mM vanadyl ribonucleosides complexes

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Table 1. Description of probes

Probe	Plasmid	5′, 3′ bp	Size (bp)	References
IL-1	pMIL-1	(−46, 606)	652	21
IL-2	pMIL-2	(297, 443)	146	22
IL-4	pSP6K	(−61, + 181)	725	23
IL-6	pUC8-IL-6	(1, 1089)	1089	24
IFN-γ	pATM-IFN	(270, 581)	311	25
TNF-α	pGEM-TNF	(20, 1664)	1644	26

(VRC; Sigma) and 5 mM $MgCl_2$ for 15 min at room temperature. After washing, slides were treated with 0.25% Triton X-100 (Sigma) in PBS containing 20 mM VRC and 5 mM $MgCl_2$ for 10 min at room temperature, dehydrated and stored at 4°C in 70% ethanol.

Preparation of probes

All the probes used for this study are listed in the Table 1; their specificity was described previously (21–26). These

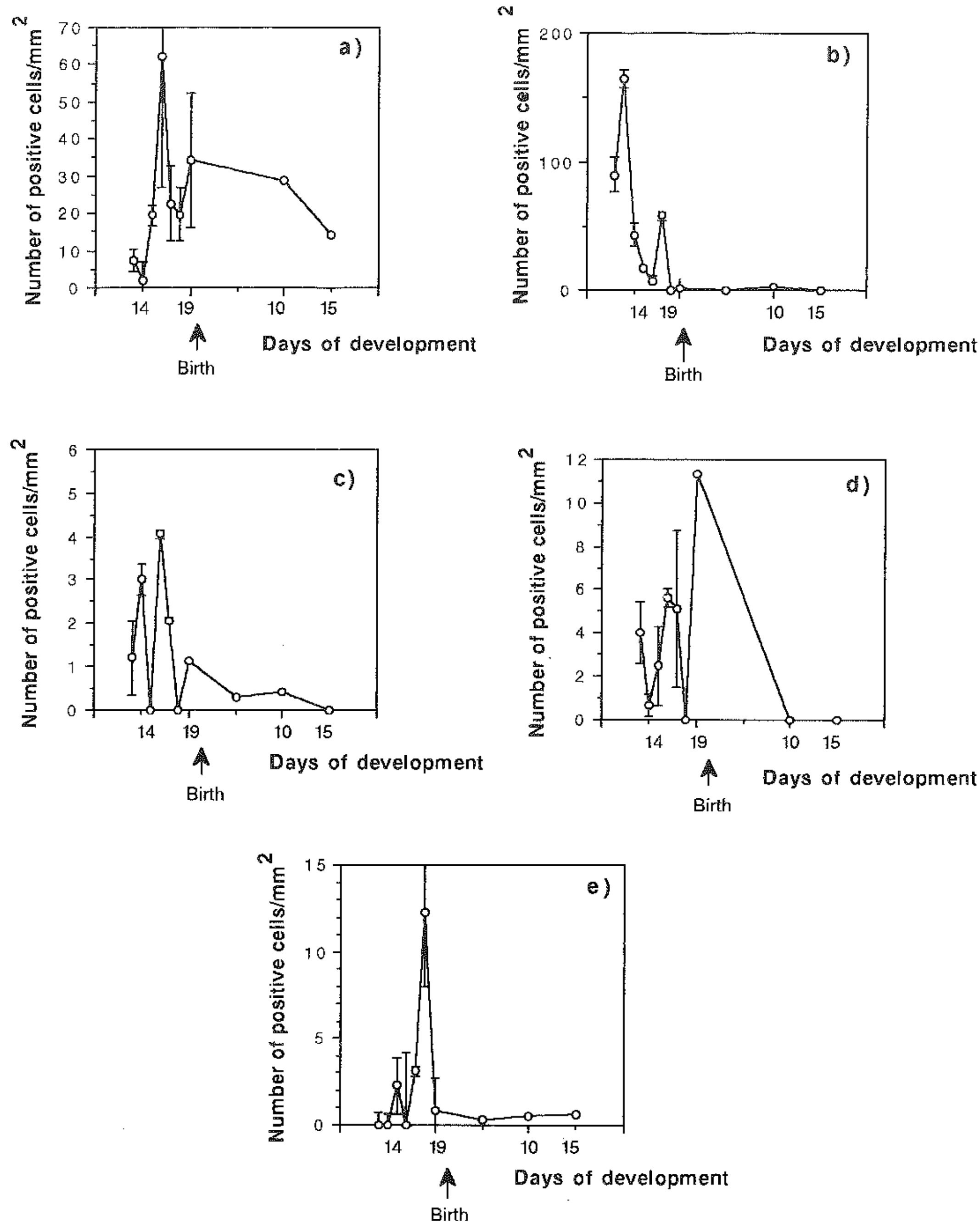

Fig. 1. Density of thymic cells producing IL-1 (a), IL-2 (b), IL-4 (c), IL-6 (d) and IFN-γ (e) mRNAs (arrows) during ontogeny (as assessed by RNA–RNA *in situ* hybridization on cryosections). Means ± SE from four independent experiments are shown.

DNA probes were transferred by cloning into p-GEM (27). Linearized plasmids were used as templates for the *in vitro* synthesis of RNA probes complementary to the cellular mRNA (antisense probe). RNAs were also transcribed in the opposite direction (sense probe) and used as a negative control. These probes were labeled by *in vitro* transcription using SP6 or T7 RNA polymerases and ^{35}S-labeled UTP and CTP according to the supplier's recommendations (Boehringer Mannheim, Mannheim, Germany). The specific activity was ~1×10^8 c.p.m./mg.

In situ hybridization

Fifty microliters of probe mixture of 50% formamide containing NaCl (0.6 M), Tris–HCl (10 mM), EDTA (1 mM), 1% SDS, dithiothreitol (10 mM), yeast t-RNA (0.25 mg/ml) (Boehringer Mannheim), 1×Denhardt's (50×Denhardt's = 1% Ficoll, 1% polyvinylpyrrolidine, 1% BSA), 10% PEG-6000 and 1 ml of labeled RNA (150,000 c.p.m./ml) was loaded on each slide, and hybridization was performed at 50°C overnight in a humid chamber. As a negative control, a few slides were pretreated for 2 h at 37°C in a solution containing 20 mg/ml of RNase to destroy the mRNA or the antisense probe was replaced by the sense probe. Slides were then washed twice for 5 min with PBSM (PBS containing 5 mM $MgCl_2$), rinsed for 30 min in a solution containing 20 mg/ml of RNase (Boehringer Mannheim) in 0.5 M NaCl and 10 mM Tris-HCl (pH 8) at 37°C, 30 min in the same solution except RNase at 37°C, 30 min with 50% formamide/2×SSC (2×SSC = 0.3 M NaCl/0.03 M sodium citrate) at 50°C, 30 min with 50% formamide/1×SSC and 30 min in 50% formamide/1×SSC containing 0.05% Triton-X at 37°C. The slides were then dehydrated successively in 30, 50 and 70% ethanol in 300 mM ammonium acetate (pH 7.0), air dried and finally autoradiographed. Ilford K2 emulsion was diluted with an equal volume of 300 mM ammonium acetate. The slides were dipped into the emulsion and allowed to solidify horizontally at room temperature for 4 h. The emulsion-coated slides were kept at 4°C for 8–10 days for exposure. They were developed in Kodak D-19 developer (Eastman Kodak, Rochester, NY) for 3 min. After a rinse in a 1% acetic acid solution, the fixation was carried out in an Ilford rapid fixer for 6 min and the slides were washed twice with water for 30 min. They were stained with hematoxylin & eosin for 2 min, washed twice with water and air dried. We considered as positive cells (cells expressing mRNAs) those cells that had more than eight grains per cell (Fig. 2D).

The anatomical distribution of positive cells was observed on cryosections and their density was estimated as follows: the total number of labeled cells was counted for the whole of each section using a light microscope (Leitz) and the section area was estimated with an image analyzer 'IBAS' (J.-M. Paulus, Laboratory of Hematology). The number of positive cells was then evaluated by unit of area. The percentages of labeled cells were also estimated on cytocentrifuge preparations.

These experiments were performed independently four times.

Statistical analysis

The data were analyzed using the Student 's *t*-test with transformed values (StatWork; Cricket Software).

Table 2. Frequency of thymic cells producing cytokine mRNAs during ontogeny estimated on cytocentrifuge preparations

mRNA detected	Positive cells at gestational age (%)						
	13 days	14 days	15 days	16 days	17 days	18 days	19 days
IL-1	0.000	0.010	0.030	0.200	0.007	0.000	0.000
IL-2	0.000	0.085	0.000	0.12	0.01	0.096	0.000
IL-4	0.001	0.002	0.000	0.001	0.0015	0.000	0.000
IL-6	0.120	0.010	0.014	0.000	0.000	0.000	0.070
TNF-α	NT	0.000	0.023	0.008	0.003	0.003	NT
IFN-γ	0.000	0.000	0.000	0.000	0.005	0.010	0.090

NT, not tested.

Results

Production of cytokines mRNAs in the thymus during ontogeny

The chronology of cytokines mRNAs production in the fetal thymus was reproduced in four independent experiments (Fig. 1 and Table 2).

The density (Fig. 1a) and the percentage (Table 2) of positive cells for IL-1 probe progressively increased from day 14 and reached a maximum on day 16, when 0.2% of cells were labeled. Thereafter, the density of positive cells slightly decreased up to day 18 and was maintained relatively constant after birth.

Two waves of IL-2 mRNA were observed: the first one occurred on day 14, whereas the second one peaked on day 18 (Fig. 1b and Table 2).

Two peaks were also detected with the IL-4 probe (Fig. 1c and Table 2): the first one on day 14 (0.002% of positive cells), the second one on days 16–17 (0.001% of positive cells).

In the case of IL-6, three waves of mRNA were detected on cryosections (on days 13, 16–17 and around birth; Fig. 1d), whereas, on cytocentrifuge preparations, positive cells were only detected between days 13 and 15 (Table 2).

For TNF-α, the percentages of positive cells calculated on cytocentrifuge preparations (Table 2) confirm our previous results obtained on cryosections (20) with an increase of positive cells around birth.

The density (Fig. 1e) and the percentage (Table 2) of cells containing IFN-γ mRNA increased from day 17, reaching a maximum of 0.01–0.1% of labeled cells on days 18–19.

Except for cells labeled by the IL-1 (Fig. 2A) and the TNF-α (20) probe, which were generally grouped and more often localized in the outer part of the thymic lobes at the beginning of gestation and in the cortex at days19–20, when the cortex and the medulla were clearly segregated, the other positive cells (for IL-2, IL-4, IL-6 and IFN-γ) (Fig. 2B and C) were evenly distributed in the thymus.

The specificity of hybridization was assessed using probes of the sense orientation (Fig. 3A), by pretreating the sections with RNase (Fig. 3B) or by replacing the probe by water; under such control conditions, no detectable hybridization signals were seen.

Discussion

Knowledge of the time course of intrathymic cytokine production is essential to assess their intervention in the thymocyte

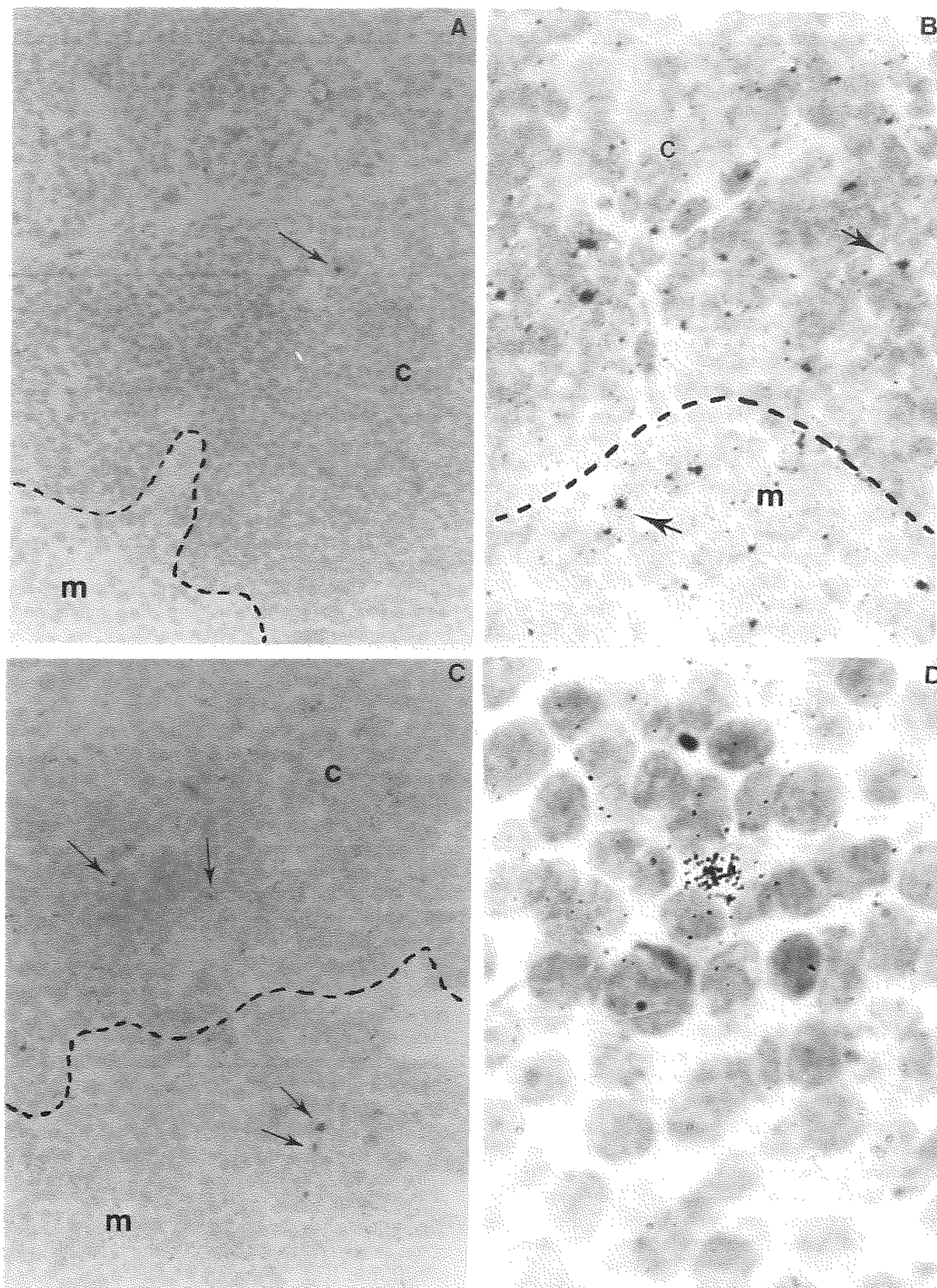

Fig. 2. Examples of detection by *in situ* hybridization of cells containing IL-1 (A), IL-2 (B) and IFN-γ (C) mRNAs in fetal thymus frozen sections at days 19 (A) and 18 (B and C) of gestation. Positive cells have more than eight grains per cells (D). IL-1 positive cells are located in the cortex (c) whereas IL-2 and IFN-γ positive cells are found in the cortex and in the medulla (m); the boundary between the cortex and the medulla is indicated by a broken line (A–C, ×420; D, ×1000).

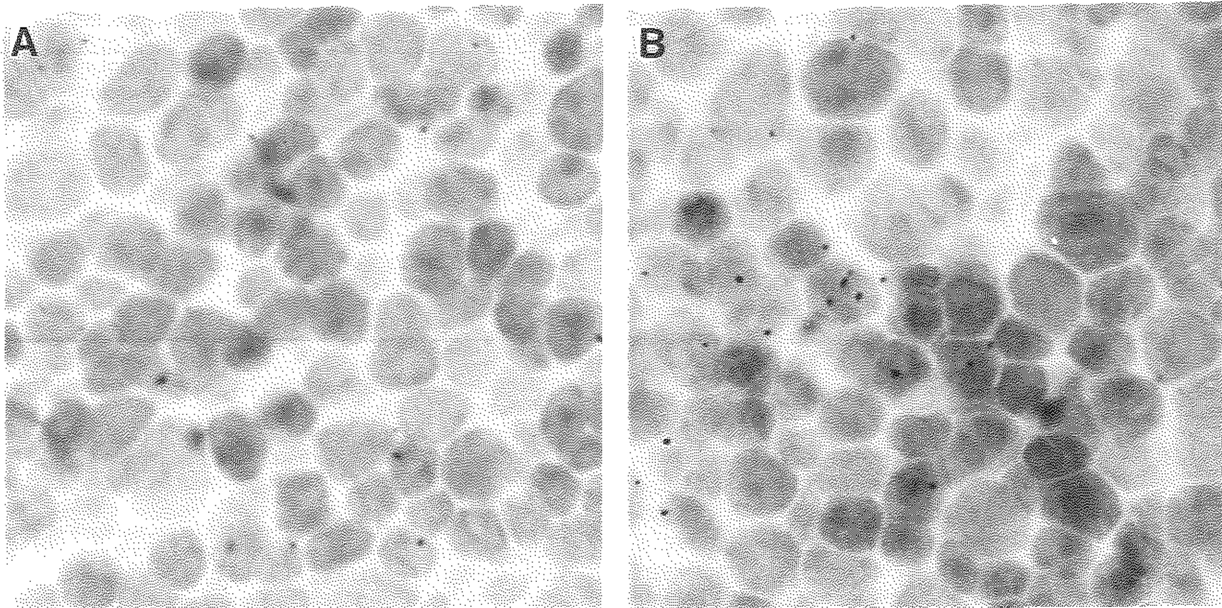

Fig. 3. Example of negative controls: no hybridization signal is seen with the control sense probe (section treated by the sense probe for IL-1) (A) or after pretreatment of the sections with RNase (B) (A and B, ×1000).

developmental program and to understand their concerted action on developing T lymphocytes. This knowledge requires the analysis of cytokine transcript expression in the thymus and the detection of cytokine synthesis. This work extends other studies indicating that the fetal thymus contains various cytokine transcripts (19,20,28–31). For the majority of the probes (IL-1, IL-2, IL-4, IFN-γ and TNF-α), the evolution of the positive cells was similar whether their frequency was estimated on cryosections or whether their percentage was calculated on cytocentrifuge preparations. However, the kinetics of IL-6 transcripts appearance were different on sections and on cytocentrifuge preparations. This could be explained by the differences in the techniques used: in studies on frozen sections there is no risk of missing some subpopulations of cells and particularly of stromal cells with cytokine transcripts, which could be lost during the preparation of cell suspensions.

This could also explain the differences between our results and those of Carding *et al.* (19) concerning the presence of IL-2 and of IL-4 transcripts. Furthermore, the mouse strains analyzed were not identical: we observed ourself differences between C57BL and BALB/c mice (data not shown), and Dallman *et al.* also described a different chronology of cytokine transcript expression in the thymus of BALB/c mice (29). These observations should be related to the variation in thymocyte development for a given gestational age with strains, e.g. day 14 fetuses of CBA and BALB/c mice and day 13 fetuses of C57BL mice were developmentally comparable (31).

Observations on frozen sections allow topographical localization of the positive cells in the thymus—this may be of importance since populations of thymocytes and stromal cells are heterogeneous within the cortex (and within the cortex, within the outer cortex versus the deep cortex) and within the medulla, and since these cells support different stages of T cell development (32). IL-1 and TNF-α transcripts are detected within the cortex: TNF-α is most probably produced by macrophages and by immature thymocytes (20), whereas IL-1 could be produced by immature thymocytes, by macrophages but also by cortical epithelial cells, as previously demonstrated by immunohistochemistry in the human thymus (9). IL-2, IL-4, IL-6 and IFN-γ mRNAs are evenly distributed. IFN-γ, IL-2 and IL-4 are generally assumed to be produced by immature cells (5,19,31), whereas epithelial cells probably participate in IL-6 production (10,11); however, the presence of these transcripts within the medulla raises the question of possible production by more mature thymocytes.

What could be the effects of cytokines upon thymocyte differentiation? As already mentioned, results obtained *in vitro* using cell suspensions seem to indicate that cytokines influence thymocyte proliferation and/or differentiation (5,12,13,16–18) and modulate some properties of the thymic microenvironment (14,15). They have also been shown either to stimulate or to inhibit thymocyte differentiation in organ cultures of thymic lobes (34–38).

During ontogenesis, the thymus, initially composed of epithelial cells (39), is progressively colonized by mesenchymal cells and by precursor cells originating from hematopoietic marrows and fetal liver. These precursors give rise to macrophages and interdigitating cells that are already present on day 14 of gestation, and to lymphocytes which are first detected in the 11 day old fetal thymus (2,40,41). Early thymocytes do not possess any essential molecule for the function of T lymphocytes, i.e. the CD3–TCR complex and CD4 and/or CD8 molecules. All these molecules will be progressively expressed according to a definite order, simultaneously with the expansion of the lymphoid compartment in the thymus (2,40,41). It is interesting to observe that the peak of IL-1 transcripts is almost simultaneous with the

expression of both CD4 and CD8 molecules (2), and also with the appearance of thymic nurse cells on day 17 of gestation (42); likewise, the emergence of single-positive cells corresponds to the presence of IL-1, IL-2 and IFN-γ transcripts on days 18–19, and with IL-6 and TNF-α transcripts around birth.

In vitro data have suggested that cytokines could play a role in intrathymic selection. IL-1 (43) or IL-2 (44,45) inhibit the anti-CD3 induced apoptosis, whereas TNF-α can induce apoptosis (Deman *et al.*, in preparation). These observations suggest that secretion or lack of secretion of some cytokines may be a physiological mechanism implicated in the process of selection.

Recently, important findings on the function of cytokines have been obtained by the construction of mice overexpressing IL-4 (46) or lacking IL-2 (47) or IL-4 (48) gene expression. On the one hand, increased intrathymic expression of IL-4 significantly perturbs the development of $CD4^+CD8^+$ thymocytes (46) and, on the other hand, T cell development is normal in mice homozygous for a mutation that inactivates the IL-4 gene (48) or in IL-2 gene deficient mice (47) as well as in $IL\text{-}2^{-/-}$ $IL\text{-}4^{-/-}$ mice (49), suggesting that neither IL-2 nor IL-4 is essential in T cell development.

Other indications concerning the functions of cytokines have been obtained after intrathymic transfer of precursor populations and administration of cytokines in radiochimeras (50): these studies indicated that recombinant IL-6 (rIL-6) accelerated the differentiation of $CD3^-CD4^-CD8^-$ precursors into $CD4^+CD8^+$ and $CD4^+CD8^-$ subsets, whereas rIL-2 blocked their normal differentiation and expansion. Our observation that, during gestation, two important peaks of mRNAs for IL-6 are detected, the first one at days 16–17, simultaneously with the appearance of double-positive cells, and the second one around birth, when $CD4^+CD8^-$ cells develop, encourages the view that IL-6 plays a role in the differentiation of precursors. Moreover, the expression of IL-2 mRNA strongly decreased between days 15 and 17 of gestation, a period during which there is an increase in the proportion of $CD4^+CD8^+$ thymocytes whose development is blocked by IL-2.

This report demonstrates the presence of cytokine transcripts in the developing thymus following a developmentally controlled sequence. However, questions remain to be solved in the future concerning the role of cytokines in T cell development. One important step to answer these questions will be the intrathymic detection of cytokines at the protein level and the identification of cytokine producing cells. These experiments are presently in progress.

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Abbreviations

TNF	tumor necrosis factor
VCR	vanadyl ribonucleosides complexes

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