

The IL-2/IL-2-Receptor Complex in the Maturation of Rat T-Cell Progenitors

ALBERTO VARAS, TERESA ROMO, EVA JIMÉNEZ, LUIS ALONSO, ANGELES VICENTE
and AGUSTÍN G. ZAPATA*

Department of Cell Biology, Faculty of Biology, Complutense University, 28040 Madrid, Spain

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On the basis of both the interleukin-2-receptor (IL-2R) α -chain expression on 16-day-old fetal rat thymocytes and the occurrence of interleukin-2 (IL-2) mRNA-containing cells early during rat thymus ontogeny, we have investigated the possible role of IL-2/IL-2R complex in rat T-cell maturation. For this purpose, we analyzed the effects of the addition of either recombinant rat IL-2 or anti-CD25 (OX-39)-blocking monoclonal antibodies to fetal thymus organ cultures (FTOC), established from 16-day-old rat embryos. IL-2 stimulated the growth of thymocytes and, as a result, induced T-cell differentiation, whereas OX-39 mAb blocked the maturation of thymic-cell progenitors. Accordingly, these results support the involvement of IL-2/IL-2R complex in rat T-cell development.

Keywords: Interleukin-2 (IL-2), interleukin-2 receptor (IL-2R), rat, thymocytes

INTRODUCTION

It is now accepted that interleukin-2 (IL-2) is a potent growth factor for mature T cells. However, much controversy has arisen concerning its role, if any, in the first stages of T-cell development. The expression of individual chains (α , β , and γ) of the IL-2 receptor (IL-2R) on immature thymocytes (Ceredig et al., 1985; Toribio et al., 1989; Kondo et al., 1994; Reya et al., 1996), as well as the ability of these cell subsets to produce IL-2 (Tentori et al., 1988b; Zlotnik et al., 1992) and proliferate in its presence (Ceredig et al., 1989; Toribio et al., 1989; Brooks et al., 1993),

support the idea that IL-2 may drive the proliferation and the differentiation of T-cell precursors. In fact, the culture of T-cell progenitors with IL-2 promotes their differentiation to TcR $\alpha\beta$, TcR $\gamma\delta$, and NK cells (Toribio et al., 1988; De la Hera et al., 1989; Brooks et al., 1993; He and Kabelitz, 1995), and *in vivo* or *in vitro* treatments that alter the IL-2/IL-2R complex profoundly modify the T-cell maturation (Jenkinson et al., 1987; Skinner et al., 1987; Tentori et al., 1988a; Plum et al., 1990; Waanders and Boyd, 1990; Zuñiga-Pflücker and Kruisbeek, 1990; Zuñiga-Pflücker et al., 1990; Kroemer et al., 1991; Maslinski et al., 1992). Furthermore, two waves of IL-2 mRNA production,

*Corresponding author.

which correlate well with the differentiation of two waves of T-cell precursors (Jotereau *et al.*, 1987; Penit and Vasseur, 1989), have been reported during fetal thymus development (Montgomery and Dallman 1991; Deman *et al.*, 1994). Nevertheless, gene-disruption experiments suggest that IL-2/IL-2R complex is not required for the generation of normal cell populations in the murine thymus (Schorle *et al.*, 1991; Suzuki *et al.*, 1995; Willerford *et al.*, 1995). In addition, some authors reported the lack of CD25 expression on rat immature thymocytes (Takacs *et al.*, 1988; Kampinga and Aspinall, 1990). However, we conclusively demonstrated the expression of IL-2R α chain on CD4⁻CD8⁻CD3⁻ triple-negative (TN) cells during rat thymus ontogeny. From this basis, we have analyzed the effects of the addition of recombinant rat IL-2 and the blockade of IL-2R by anti-CD25 antibodies on rat thymocyte maturation, using fetal thymus organ cultures (FTOC) established from fetal day-16 thymic lobes.

RESULTS

Expression of IL-2 Receptors and IL-2 mRNA on Rat Fetal Thymocytes

The flow cytometrical analysis of rat fetal thymocytes demonstrated that the highest proportion of IL-2R α -expressing thymocytes (20-30%) occurred at day 16 of gestation, when most thymocytes (~90%) corresponded to CD3⁻CD4⁻CD8⁻ cells (Figure 1a). At the same stage, 20-25% of total thymic cells contained IL-2 mRNA, as detected by *in situ* hybridization (Figure 1b).

Thymocyte Development in Rat FTOC

According to the acquisition of CD4, CD8, and TcR $\alpha\beta$ cell markers, rat FTOC mimicked the *in vivo* T-cell development during rat thymus ontogeny. In the first 4 days of culture, there was a gradual decrease of the frequency of CD4⁻CD8⁻ double-negative (DN) cells, in correlation with the appearance of immature CD4⁻CD8⁺, CD4⁺CD8⁺ double-positive (DP), and mature CD4⁺CD8⁻ and CD4⁻CD8⁺ single-positive (SP) thymocytes (Figure

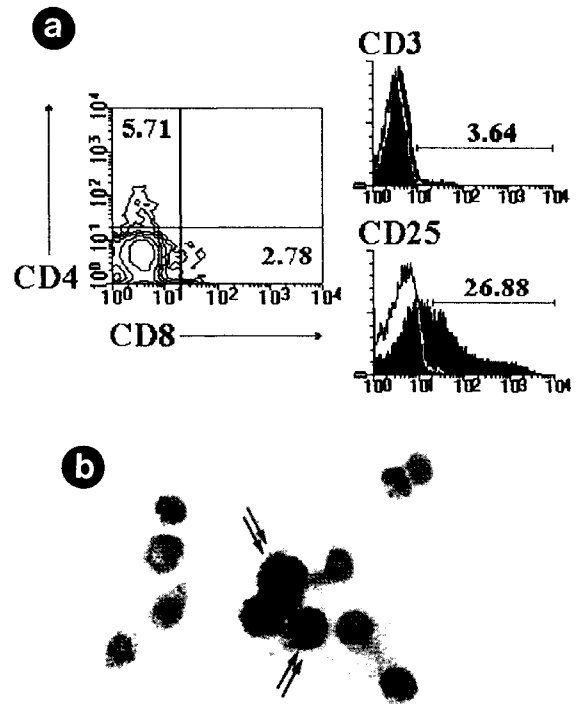


FIGURE 1 (a) Expression of CD4, CD8, CD3, and CD25 antigens on fetal thymocytes from 16-day-old rat embryos. (b) IL-2 mRNA-containing cells (arrows) in thymocyte cytospin preparations from 16-day-old fetal rats.

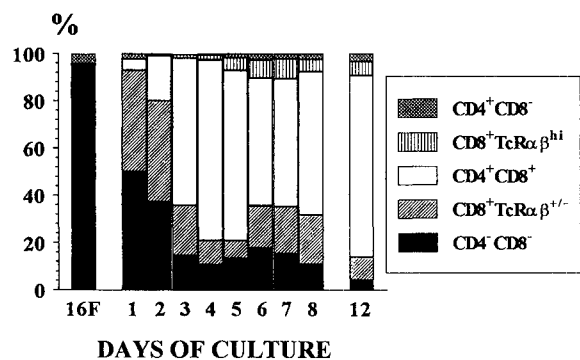


FIGURE 2 Evolution of control rat FTOC. Percentages of the different thymic-cell subsets defined according to the coordinate expression of CD4 and CD8. In these analyses, the subset CD4⁻CD8⁺ was subdivided into a CD8⁺TcR $\alpha\beta$ ^{high} cell subpopulation, obtained from gates in double stainings of CD8/TcR $\alpha\beta$, and a CD8⁺TcR $\alpha\beta$ ^{low} cell subset, corresponding to immature CD8⁺ cells and CD8⁺TcR $\gamma\delta$ thymocytes.

TABLE I CD25 Expression on CD3⁻ Thymocytes in Rat Fetal Thymus Organ Cultures

Day 1	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 12
6.72 ± 2.03	5.40 ± 0.67	3.92 ± 0.60	7.43 ± 1.33	5.55 ± 0.71	3.92 ± 0.53	4.58 ± 0.67	6.66 ± 0.60

Note: At different times of culture, thymocytes were stained with anti-CD25 and anti-CD3 antibodies, and the expression of CD25 was analyzed in the CD3⁻ cell compartment. Data are expressed as the mean ± SEM from five to seven independent experiments.

2). As occurs *in vivo* (Vicente et al., manuscript submitted), in those days of culture (5 to 7) equivalent to the perinatal period, a new signal of expansion occurred in rat FTOC, inducing a transient increase of the DN-cell subset and its differentiation to immature CD8⁺, DP, and mature SP thymocytes in the following days (Figure 2). At that time, the percentage of CD25⁺, which had gradually diminished during the first 4 days of culture, sharply increased also in the CD3⁻ cell compartment (Table I).

Effects of IL-2 on the Development of Thymic Major Cell Subpopulations

The number of cells per thymic lobe increased after 1 day of treatment with IL-2, remaining unchanged at day 3 and even decreasing after 5 to 7 days of culture. However, one more week of culture in the presence of IL-2 increased again the thymic cellularity (Figure 3).

One day of culture with IL-2 basically induced an increase in the numbers of DN and CD4⁺ cells (Figure 3). At day 3, the absolute numbers of the different thymocyte subsets hardly changed, excepting for the slight increase in mature CD8⁺ thymocytes. This cell subset remained unaltered after 5 days of treatment, whereas the cell numbers of the rest of thymocyte subpopulations decreased (Figure 3). By day 7 of culture, the number of DN cells reached control values and that of mature CD8⁺ thymocytes increased. Five more days of treatment with IL-2 induced a cell expansion affecting to all thymocyte subsets, but in a higher proportion to the DN-cell subpopulation (Figure 3).

Effects of Anti-CD25 Treatment on Thymocyte Maturation

Anti-CD25 treatment was carried out by adding OX-39 mAb, known by blocking the binding of IL-2 to

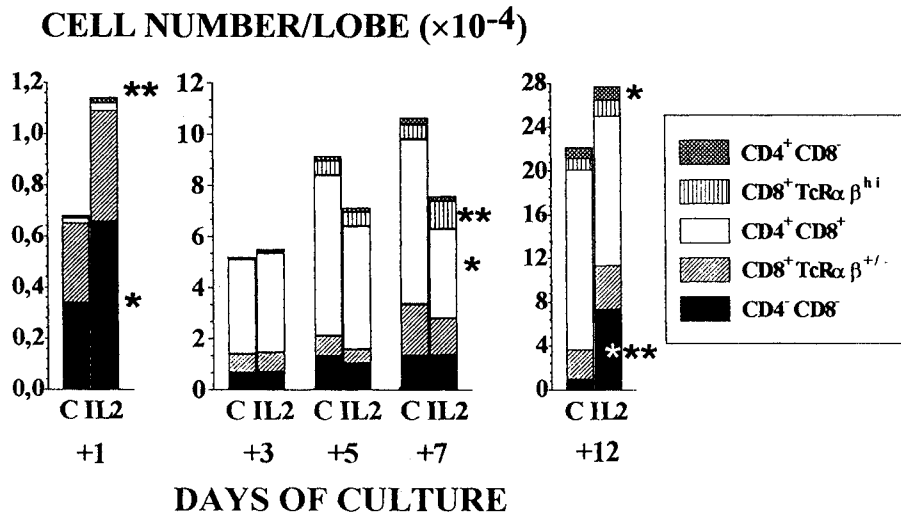


FIGURE 3 Absolute numbers of different thymic-cell subpopulations in control (left bar) and IL-2-treated (right bar) FTOC for 1, 3, 5, 7, and 12 days. Thymocyte subsets were defined in double stainings by expression of CD4, CD8 and TcRαβ. At each time point, the data represented are the mean of three to five independent experiments. * p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 (Student's t test).

TABLE II Cell Number of Thymocyte Subsets after Anti-CD25 Treatment

Time		Phenotype					Total
		CD4 ⁻ CD8 ⁻	CD8 ⁺ TcR $\alpha\beta$ ^{+/} -	CD4 ⁺ CD8 ⁺	CD8 ⁺ TcR $\alpha\beta$ ^{high}	CD4 ⁺ CD8 ⁻	
Day 1	Control	0.58 ± 0.11	0.50 ± 0.09	0.06 ± 0.01	—	0.03 ± 0.01	1.17 ± 0.22
	OX-39	0.35 ± 0.07	0.21 ± 0.04 ^b	0.02 ± 0.01 ^a	—	0.02 ± 0.00	0.60 ± 0.12 ^a
Day 3	Control	0.63 ± 0.06	1.03 ± 0.10	2.94 ± 0.38	0.08 ± 0.01	0.01 ± 0.00	4.69 ± 0.55
	OX-39	0.61 ± 0.06	0.76 ± 0.12 ^a	1.18 ± 0.32 ^b	0.06 ± 0.01	0.01 ± 0.00	2.62 ± 0.52 ^b
Day 5	Control	1.10 ± 0.30	0.66 ± 0.22	6.46 ± 1.43	0.43 ± 0.07	0.13 ± 0.06	8.78 ± 2.10
	OX-39	0.63 ± 0.26	0.14 ± 0.02 ^b	2.74 ± 0.86 ^b	0.08 ± 0.02 ^c	0.02 ± 0.01 ^a	3.61 ± 1.12 ^b
Day 7	Control	1.20 ± 0.18	1.92 ± 0.30	4.41 ± 1.22	0.93 ± 0.20	0.30 ± 0.12	8.76 ± 1.73
	OX-39	0.88 ± 0.19	0.42 ± 0.11 ^b	0.62 ± 0.16 ^b	0.15 ± 0.03 ^c	0.06 ± 0.01 ^b	2.13 ± 0.41 ^c
Day 12	Control	0.76 ± 0.16	2.22 ± 0.44	20.05 ± 4.64	1.90 ± 0.52	1.01 ± 0.30	25.94 ± 6.08
	OX-39	0.46 ± 0.15	0.21 ± 0.07 ^c	0.62 ± 0.25 ^c	0.08 ± 0.03 ^c	0.03 ± 0.02 ^c	1.40 ± 0.55 ^c

Note: Values represent the mean cell number ($\times 10^{-4}$) per lobe \pm SEM from three to six independent experiments.

^a $p \leq 0.05$;

^b $p \leq 0.01$;

^c $p \leq 0.001$ (Student's *t* test).

high-affinity IL-2 receptors (Paterson *et al.*, 1987; Somoza *et al.*, 1990).

From the beginning of culture, the addition of OX-39 mAb markedly inhibited viable cell yield in rat FTOC. The reduction in thymic cellularity was increasing to reach the highest effect after 12 days of treatment (Table II).

In the continuous presence of OX-39, the absolute numbers of all thymocyte subsets were always lower than in control FTOC, being, at any time, the DN-cell subpopulation the least affected by the treatment (Table II). However, whereas the differentiation of the second wave of T-cell progenitors was totally inhibited, the cell precursors present in 16-day-old fetal thymus partially matured, presumably because some of them had already overcome the CD25⁺ stage (Table II).

DISCUSSION

The current data indicate that rat T-cell precursors (TN cells) express IL-2 receptors, a fact previously denied by other authors (Takacs *et al.*, 1988; Kampinga and Aspinall, 1990), although repeatedly reported in mice, chickens, and humans (Ceredig *et al.*, 1985; Toribio *et al.*, 1989; Zuñiga-Pflücker *et al.*, 1990; Feddecka-Brunner *et al.*, 1991). In agreement with our results, Brocke *et al.* (1987) found IL-2R α -

bearing cells in the thymus of 16-day-old fetal rats. However, the immunohistological demonstration of CD4 and CD8 expression in these thymic lobes was misinterpreted, concluding that CD25⁺ thymocytes corresponded to mature thymocytes. Obviously, as our results demonstrate, the rat thymic primordium does not contain mature SP thymocytes but DN cells, some of which, in progression to the DP-cell subset, could already express CD4 and/or CD8 molecules in their cytoplasm.

On the other hand, since IL-2 supports the growth of rat thymocytes in FTOC, in a way that can be blocked by anti-CD25 antibodies, IL-2 receptors expressed on rat fetal thymocytes seem to be functional. Contradictory results have been reported on the ability of IL-2 to induce proliferative responses in early thymocytes (Raulet, 1985; von Boehmer *et al.*, 1985; de la Hera *et al.*, 1989; Toribio *et al.*, 1989; Brooks *et al.*, 1993). Presumably, as recognized by many authors, this induction is dependent on thymic stromal cells or an intact thymic microenvironment, as provided by organ cultures (De la Hera *et al.*, 1989; Ceredig *et al.*, 1989; Zuñiga-Pflücker *et al.*, 1990). However, the continuous addition of IL-2 to rat FTOC inhibits thymocyte growth and T-cell maturation, as also reported in mouse organ cultures (Skinner *et al.*, 1987; Plum *et al.*, 1990; Waanders and Boyd, 1990). The generation of LAK cells has been pointed out to be involved in the depletion of thymocytes and the

subsequent inhibition of T-cell differentiation (Skinner et al., 1987). Alternatively, negative signals transduced via the IL-2R in response to the IL-2 concentrations used in these experiments, which are not continuously present *in vivo*, could also explain these results (Waanders and Boyd, 1990). In fact, the inhibition of cell proliferation (Suwa et al., 1995) and the induction of apoptosis (Lenardo, 1991; Migliorati et al., 1993) have been reported after IL-2 treatment.

Our results obtained after IL-2 and anti-CD25 treatments, in agreement with previous findings in both humans and mice (Toribio et al., 1988; Zuñiga-Pflücker and Kruisbeek, 1990; Wilson et al., 1994) also indicate that IL-2 promotes the differentiation of thymic-cell precursors, as a consequence of its capacity to stimulate cell proliferation.

Taken together, these results support a role for the IL-2/IL-2R complex in the intrathymic maturation of rat T-cell precursors.

MATERIALS AND METHODS

Animals

Wistar rats were maintained in our animal facilities. Fetuses at day 16 of gestation were obtained from timed pregnancies. The day of finding a vaginal plug was designated day 0 of gestation.

Fetal Thymus Organ Cultures

Thymic lobes were aseptically removed from 16-day-old rat embryos, trimmed of surrounding mesenchyme, and organ-cultured as follows. Four to six thymic lobes were placed on the surface of polycarbonate filters (Millipore Ibérica, Spain) supported by stainless steel screen pieces. Lobes were cultured in the central well of organ tissue culture dishes (Becton-Dickinson, Spain) with 1 ml of RPMI 1640 medium (2 mM L-glutamine) supplemented with sodium pyruvate (1 mM), streptomycin (100 µg/ml), penicillin (100 U/ml) (all reagents: Gibco BRL, France), and 10% FCS (Biosys, France). The cultures were grown in a humidified incubator in 10% CO₂ in air at 37°C, and the medium was replaced daily. The

control cultures were done as described, the IL-2-treated organ cultures were performed at a concentration of 20 U/ml of recombinant rat IL-2 (Serotec, UK), and the OX-39-treated cultures were carried out in complete medium supplemented with 50% culture supernatant from OX-39 hybridoma (anti-rat CD25). In this case, control cultures made in parallel were supplemented with 50% culture supernatant from an irrelevant hybridoma (OX-14, anti-rat IgG2b).

Cell-Surface Staining

At various times through the culture period, a cell suspension was made of thymic lobes, total cell count was done, and the expression of CD4 (OX-38-PE), CD8 (OX-8-FITC), TcRαβ (R. 73-PE), CD3 (G4.18-FITC), and CD25 (OX-39-PE) (all from Pharmingen, USA) were analyzed. Flow cytometric analysis was carried out on a FACScan (Becton-Dickinson, USA). Dead cells were excluded from data acquisition on the basis of forward/side scatter and propidium iodide staining.

In Situ Hybridization

Fetal thymocytes from day 16 of gestation were isolated, cytospun onto slides, and fixed in paraformaldehyde (4% in PBS) during 30 min at room temperature. After permeabilization with proteinase K (2 µg/ml) in Tris-EDTA during 20 min at room temperature, cells were acetylated and dehydrated. Hybridization was carried out at 37°C in 5× SSC, 30% formamide, herring DNA (10 mg/ml), t-RNA (10 mg/ml), and 5 µg/ml of digoxigenin-labeled cDNA probe for rat IL-2. Slides were washed in 30% formamide in 2× SSC at room temperature and 42°C during 5 and 15 min, respectively. Anti-digoxigenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) were used for the immunological detection according to the commercial supplier's recommendations.

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