Early Maturation of T-Cell Progenitors in the Absence of Glucocorticoids

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In the present work, we demonstrated that both fetal liver and thymic T-cell precursors express glucocorticoid receptors (GRs) indirectly suggesting a role for glucocorticoids (GCs) in the earliest events of T-cell differentiation. To evaluate this issue, we analyzed the thymic ontogeny in the progeny of adrenalectomized pregnant rats (Adx fetuses), an in vivo experimental model, which ensures the absence of circulating GCs until the establishment of the fetal hypothalamus-pituitary-adrenal (HPA) axis. In the absence of maternal GCs, T-cell development was significantly accelerated, the process being reversed by in vivo GC replacement. Mature single positive thymocytes (both CD4 and CD8) appeared in 16-day old fetal Adx thymus when in the control fetuses, most thymocytes still remained in the double-negative (DN) $CD4^-CD8^-$ cell compartment. In addition, emigration of T-cell receptor (TcR)αβ positive cells to the spleen also occurred earlier in Adx fetuses than in control ones. In vitro recolonization of cultured deoxiguanosine-treated mouse fetal thymus lobes with 13-day-old fetal liver cell suspensions from both Adx and control fetuses demonstrated changes in the developmental capabilities of fetal liver T-cell precursors from embryos grown in the absence of GCs. Furthermore, a precocious lymphoid colonization of the thymic primordium from Adx fetuses was evidenced by ultrastructural analysis of both Adx and Sham early thymus. Both findings accounted for the accelerated T-cell differentiation observed in Adx fetuses. Together, these results support a role for GCs not only in the thymic cell death, but also in the early steps of T-cell differentiation.

THE IN VIVO INVOLVEMENT of glucocorticoids (GCs) in the normal development of mammalian embryos has been demonstrated by disruption of the GC receptor (GR) gene[1] and, indirectly, by the presence of GR in most fetal tissues.[2] In the earliest stages of development, until the establishment of the hypothalamus-pituitary-adrenal gland (HPA) axis, the maternal adrenal glands are the only source of circulating GCs for fetuses.[3,4] In this regard, Muglia et al[5] demonstrated that mating of heterozygous mice for a null corticotropin-releasing hormone (CRH) allele, with preservation of their normal maternal supply of corticosterone, produces viable homozygous CRH-deficient mice with normal growth, fertility, and longevity, despite the low levels of hormone produced by their own adrenal glands during adulthood. On the contrary, littermates from homozygous CRH-deficient mothers die within the first 12 hours of life.

The role of GCs in the development of distinct blood cell lineages, a complex process that entails the regulation of an intricate network of genes, is poorly known. GCs seem to be involved in the decision of erythroblast between self-renewal and differentiation.[6] Accordingly, the blocking of GR binding to DNA impairs the long-term proliferation of erythroid progenitors.[7] In contrast, these steroids appear to have a different effect on B-cell maturation. Corticosterone in vivo reduces the cycling B-cell precursors and induces their apoptosis, giving rise to a drastic decrease in the number of developing B-lineage cells in the bone marrow.[8] Interestingly, in vitro GCs shift the balance of granulocyte versus macrophage formation at early stages of precursor cell differentiation[9] and prevent the T-cell–mediated terminal maturation of an epidermal-derived dendritic cell (DC) line.[10]

The involvement of GCs in the generation of T-cell repertoire has been repeatedly invoked,[11-14] but their role in early T-cell differentiation has been little studied. King et al[15] suggested that GCs are necessary for the normal progression of DN ($CD4^-CD8^-$) to the double-positive (DP) $CD4^+CD8^+$ cell compartment. However, other in vivo experimental studies indicate that total or partial blockage of GC signaling has no relevant effects on adult mouse thymus.[1,7] In an attempt to clarify the role of GCs in the early T-cell maturation, we studied the thymocyte development in the progeny of adrenalectomized pregnant rats, an experimental model that ensures the absence of circulating GCs during early embryonic development, previously used by our group.[16,17] Our results demonstrate an in vivo accelerated T-cell maturation in these experimental conditions largely due to both changes of the maturation capabilities of fetal liver lymphoid precursors and a precocious lymphoid colonization of the fetal thymic primordium.

MATERIALS AND METHODS

Animals and treatment. Wistar rats and Swiss mice were maintained in our laboratory facilities. Rat and mouse fetuses were obtained from timed pregnancies. The day of finding of a vaginal plug was designated day 0 of gestation. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health (NIH).

Surgery procedure. Wistar rats were either adrenalectomized or sham adrenalectomized on the first day of pregnancy. From this moment, the pregnant rats were transferred to individual cages. Bilateral adrenalectomy (Adx) or sham adrenalectomy (Sham) was performed using the dorsal approach under ether anesthesia. The Adx mothers received 0.9% NaCl to drink instead of water until sacrifice. To reconstitute the fetal circulating corticosterone levels, 1 osmotic

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minipump (2ML4, ALZET; Alza Corp, Palo Alto, CA) was subcutaneously implanted in pregnant rats during adrenalectomy surgery procedure. The osmotic minipumps were prepared following instructions supplied by the commercial distributor to continuously infuse 23 μg/hour of corticosterone (Sigma Chemical, St Louis, MO) in a volume of 0.25 μL of propylene glycol in 0.9 % NaCl (1:1).

Corticosterone levels. The blood samples were collected in nonheparinized tubes and after 4 hours at room temperature centrifuged at 2,200 rpm for 15 minutes at 4°C. Sera were stored at −70°C until assayed. Steroid extraction using methylene chloride was performed before testing. A double antibody commercial radioimmunoassay (RIA) kit (Gamma-B-125I-Corticosterone RIA; IDS, Boldon, UK), which provides a highly sensitive method (0.04 ng/mL), was used for the determination of serum corticosterone levels from both mothers and fetuses.

GR analysis. The GR expression was analyzed on both fetal liver cell precursors and early immature thymocytes. For the study of fetal liver precursors, $CD45^+$ cells were isolated from liver cell suspensions of 13-day-old rat fetuses. Briefly, cell suspensions were stained with a fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (MoAb) against rat CD45 (OX1, Pharmingen, San Diego, CA) and $CD45^+$ cells sorted with a FACStar plus (Centro de Citometría de Flujo y Microscopía Confocal, UCM, Madrid, Spain). Sorted fetal liver $CD45^+$ cells and total thymic cells from 15-day-old rat fetuses were cytospun, fixed in acetone for 5 minutes at −20°C, and incubated with a biotin-conjugated MoAb specific for rat GR (BuGR2) (ABR, Golden, CO) for 1 hour followed by avidin-Texas red staining for 45 minutes (Amersham Iberica S.A., Madrid, Spain). An isotype-matched irrelevant antibody was used as negative control to define background fluorescence. The cells were observed and photographed under a fluorescence Labophot-2 microscope (Nikon, Tokyo, Japan).

Flow cytometry. Cell suspensions were stained with specific MoAbs during 15 minutes in phosphate-buffered saline (PBS)/2% fetal calf serum (FCS) at 4°C. Labelled MoAbs with either phycoerythrin (PE), FITC, or Cychrome against rat CD4 (OX38), CD8 (OX8), T-cell receptor (TcR)αβ (R73), CD45 (OX1) were obtained from Pharmingen. Cytometry analysis was performed in a FACScan, (Becton Dickinson, San Jose, CA) from the ''Servicio Común de Investigación'' (Faculty of Biology, UCM, Madrid, Spain). Debris and dead cells were excluded from the analysis by forward and side scatter gating, and in most cases, 10,000 to 30,000 thymocytes or splenocytes were scored. The data were analyzed using PC-lysis research software (Becton Dickinson).

Cell cycle and apoptosis analysis. Cell cycle analysis was performed by staining with 7-AAD (Sigma Chemicals). Briefly, cell suspensions were stained with FITC- or PE-labeled MoAbs. After washing, the cells were permeabilized with 30% ethanol (10 minutes, 4°C) and incubated with 1 mL of RNAse (1 mg/mL) (Sigma Chemicals) for 30 minutes. Finally, cells were incubated with 7-AAD (7.5 μg/mL) during 30 minutes at 5°C protected from light. Cells were analyzed by flow cytometry and the number of cycling cells was determined from individual gated populations on the basis of surface marker expression. Analysis was performed in a FACScan using Cell Fit and PC-lysis software (Becton Dickinson). In vivo thymic basal apoptosis was determined on freshly isolated thymocytes either from Sham or Adx fetuses by using the Annexin-V-Fluos kit (Boehringer, Mannheim, Germany) for detecting apoptotic cells by flow cytometry. Briefly, cells were washed with PBS-2% FCS and incubated with propidium iodide and FITC-labelled Annexin-V for 15 minutes at 4°C and immediately analyzed in a FACScan flow cytometer. In most cases, 20,000 cells were scored.

Fetal thymus organ cultures (FTOC) technique. Thymic lobes were placed on autoclaved polycarbonate membranes (Millipore, Iberica, Madrid, Spain) suspended by metal grids over the inner well of Falcon 3037 tissue culture plates. RPMI 1640 supplemented with 10% FCS (Biosys, Compiégne, France), piruvate (1 mmol/L), penicilin/streptomicicin (100 mg/mL), and glutamine (2 mmol/L) (all reagents: GIBCO-BRL, Eragny, France) was used as culture medium and replaced daily. Distilled H_2O was used in the outer well to maintain a humid environment. Organ cultures were kept at 37°C and 5% CO_2.

Recolonization assays. Alymphoid lobes were prepared by culturing thymic lobes from 15-day old fetal Swiss mice in FTOC in the presence of 1.35 mmol/L 2'deoxiguanosine (dGuo) (Sigma, Madrid, Spain) for 5 days as previously described.[18] After extensively washing, single depleted lobes were plated with 5×10^4 cells from 13-day old fetal liver in a total volume of 30 μL in Terasaki plates (Nalge Nunc International, Naperville, IL). Plates were then inverted to allow lobe and cells to combine at the bottom of the hanging drop.[19] After 48 hours, recolonized lobes were cultured in FTOC for 12 days before harvesting. Donor cells were prepared as follows: fetal livers were dissected either from Sham or Adx rat embryos and carefully disrupted. Debris were removed by filtering through a cotton mesh and viable cells determined by trypan blue exclusion. In all the experiments, fetal liver cell suspensions from both Sham and Adx fetuses were examined for OX-1 expression to determine the proportions of rat cell precursors used in the recolonization assays.

Electron microscopy. Thirteen-day-old fetuses aseptically isolated from either control Sham or adrenalectomized pregnant rats were fixed by immersion in 2.5% glutaraldehyde, buffered to pH 7.3 with Milloning's fluid, postfixed in 1% osmium tetroxide in the same buffer, and dehydrated in acetone for embedding in Araldite (Fluka Chemie AG, Neu-Ulm, Switzerland). Semithin sections stained with an alkaline solution of toluidine blue were used to identify and isolate the thymic primordia. Ultrathin sections of the selected areas were obtained with a Reichert OM-U3 ultratome (Reichert-Jung, Wein, Austria), double-stained with uranyl acetate and lead citrate, and examined and photographed with a JE0L 1010 electron microscope (Jeol, Tokyo, Japan) of the ''Servicio Común de Investigación'' (Faculty of Biology, UCM).

RESULTS

GR expression in fetal cell precursors and early thymocytes. To determine whether early cell progenitors, including T-cell precursors are able to respond to GCs as previously demonstrated for other blood lineages,[7-10] we analyzed the GR expression in both fetal liver cell precursors, included within $CD45^+$ cell population,[20-23] from 13-day-old rat fetuses and 15-day-old fetal thymocytes. As shown in Fig 1, earliest fetal liver $CD45^+$ cell precursors (Fig 1b) and immature thymocytes (Fig 1d), which have just arrived at the thymic primordium, are expressing GRs.

Effect of maternal adrenalectomy on corticosterone levels of the progeny. Because early cell progenitors, present in both fetal liver and thymus, expressed GRs, we tested the role that these steroids could exert on early T-cell maturation by performing an in vivo experimental model, maternal adrenalectomy, that ensures the GC absence in the progeny until the establishment of fetal HPA axis.[3,4] In fact, using this experimental approach, statistically significant lower values of circulating corticosterone (near to 0 ng/mL), as measured by RIA, occurred until fetal day 18 in the serum of the progeny of adrenalectomized rats (Adx fetuses) compared with control, Sham fetuses (Fig 2). After the establishment of fetal HPA axis on days 17 to 18 of fetal life, the significant differences disappeared (data not shown).

In vivo acceleration of thymocyte differentiation in Adx fetuses. In the absence of circulating GCs, the multiparametric flow cytometry analysis demonstrated an acceleration of in

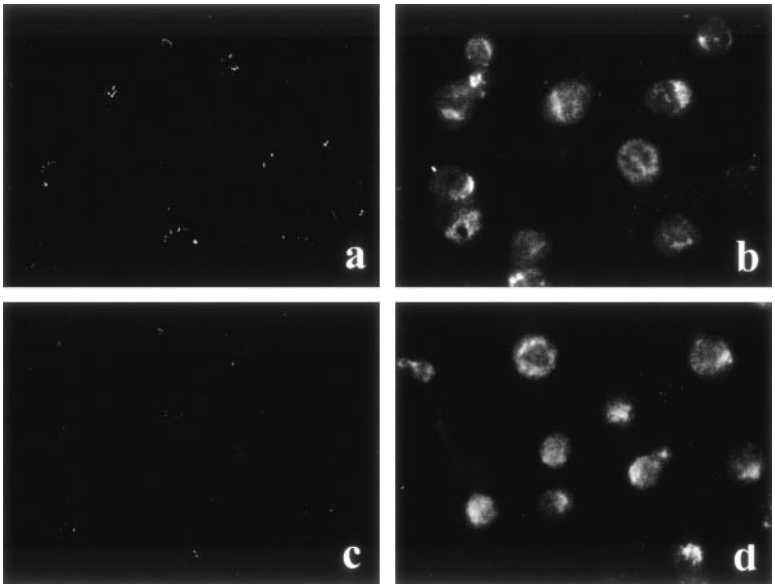

Fig 1. Glucocorticoid receptor expression in 13-day old rat fetal liver cell progenitors ($CD45^+$) (b) and 15-day old rat fetal thymocytes (d). Both sorted 13-day old fetal liver $CD45^+$ cells and 15-day old fetal total thymic cells were cytospun, fixed, and incubated with a biotin-conjugated MoAb specific for rat GR (BuGR2) followed by avidin-Texas red staining. An isotype-matched irrelevant antibody was used as negative control to define background fluorescence (a and c). The cells were observed and photographed under a fluorescence Labophot-2 microscope. Original magnification × 300.

vivo T-cell development throughout thymic ontogeny. On day 15 of fetal life, the most immature thymic population $CD4^-CD8^-TcR\alpha\beta^-$ in Sham control animals represented more than 95% of thymocytes (Fig 3A), whereas in Adx thymuses, this constituted less than 40% of cells. Moreover, the remaining cells belonged to the intermediate $CD4^-CD8^+TcR\alpha\beta^{-/lo}$ cell population (40%) and around 20% of thymocytes were DP ($CD4^+CD8^+$) cells that appeared for the first time in control Sham thymuses on day 18 of fetal life. At fetal day 15, a small proportion of thymocytes of Adx fetuses expressed, therefore, TcRαβ molecule in their membranes (Fig 3B), whereas in control Sham animals, all of the cells were $TcR\alpha\beta^-$ until day 18 of fetal life.[24] One day later, on fetal day 16, in Adx thymuses most cells had reached the DP ($CD4^+CD8^+$) stage and there was even an important percentage of mature SP (both $CD4^+CD8^-$ and $CD4^-CD8^+$) $TcR\alpha\beta^{hi}$ cells (Fig 3A and B), lymphocyte populations which under control conditions appeared in fetal rat thymus on days 20 to 21 of fetal life (data not shown).

The absolute number of thymocytes in Adx fetuses was slightly lower on day 15 of development, but significantly reduced 1 day later. To determine the contribution of the

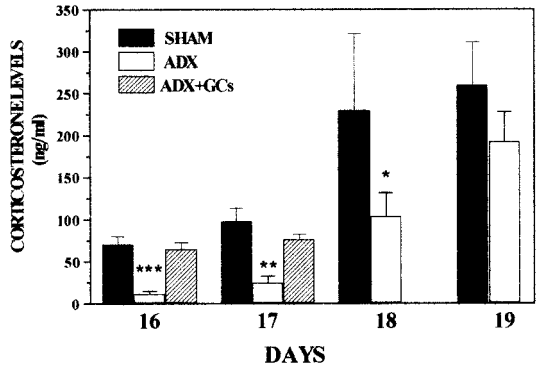

Fig 2. Circulating corticosterone levels in fetuses either from adrenalectomized pregnant rats (Adx), control, Sham rats (Sham), and adrenalectomized pregnant rats provided with corticosterone (Adx+GCs). To reconstitute the corticosterone serum levels in the Adx-fetuses, 1 osmotic minipump (2ML4, ALZET, Alza Corporation) was subcutaneously implanted in pregnant rats during adrenalectomy surgery procedure, which regularly infused 23 μg/hour of corticosterone. Data represent the average values of 3 to 4 experiments ± standard deviation (SD). Significant differences to Sham fetuses are marked as* $P \leq .05$; ** $P \leq .01$,* $P \leq .001$.**

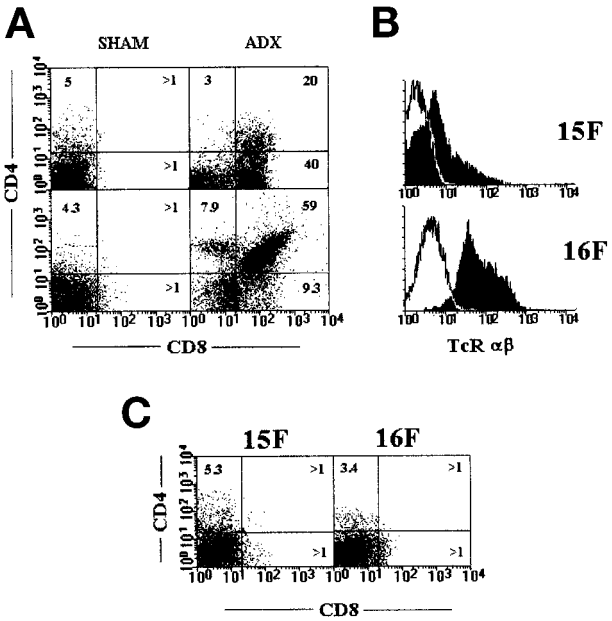

Fig 3. (A) Flow cytometry analysis of thymic T-cell populations defined by CD4/CD8 expression in 15-(up) and 16-(down) day old Sham (left) and Adx (right) fetuses. (B) Histograms represent TcRαβ expression in thymocytes from 15-(up) and 16-(down) day old Sham (open) and Adx (shadow) fetuses. (C) Thymic populations defined by CD4/CD8 expression in 15- to 16-day-old Adx fetuses from adrenalectomized mothers subcutaneously implanted with an osmotic minipump that reconstituted the corticosterone serum levels in the progeny. Results shown are representative of 3 to 4 experiments.

Table 1. Thymic Size and Percentages of Cycling and Apoptotic Cells Throughout Early Ontogeny (15 to 17 F) of Sham and Adx Fetuses

	15 F		16 F		17 F	
	Sham	Adx	Sham	Adx	Sham	Adx
Thymocyte number ($\times 10^{-4}$)	0.6 ± 0.05	0.4 ± 0.09	3.45 ± 0.25	2.0 ± 0.21*	16.3 ± 1.5	8 ± 0.6†
% Cycling cells	31 ± 3.1	30 ± 4.5	33 ± 5	27 ± 3	44.3 ± 1.4	30.3 ± 1.1*
% Apoptotic cells	4.6 ± 0.3	4.06 ± 0.5	3.56 ± 0.6	9.11 ± 2*	4.0 ± 0.7	7.5 ± 2.2*

Data represented are the average of 3 to 4 experiments ± SD.

*$P \leq .05$.

†$P \leq .01$.

‡$P \leq .001$.

intrathymic cell death and/or cell proliferation in the decrease of thymic cellularity observed in 15 and 16-day-old Adx fetuses, we analyzed, by flow cytometry, the frequency of apoptotic cells and cycling cells in both groups of embryos. No significant differences occurred in the percentages of cycling cells between control and Adx rats at fetal days 15 and 16. However, the percentage of apoptotic cells was significantly higher in the 16-day-old Adx fetuses than in control ones of the same age, as estimated by using an Annexin-V binding assay (Table 1)

On day 17, in accordance with a significant increased reduction of thymic cellularity observed in Adx fetuses (Table 1), DP ($CD4^+CD8^+$), SP ($CD4^+CD8^-$ and $CD4^-CD8^+$), and $TcR\alpha\beta^+$ thymocytes were not detected in the thymus of these fetuses (Fig 4A). Cell emigration from the thymus and/or in situ cell death could explain this finding. To examine the first possibility, flow cytometry analysis of spleen cell content of 18-day-old fetal Adx rats was performed. The study demonstrated the presence of 6% of $TcR\alpha\beta^{hi}$ splenocytes, as well as both DP ($CD4^+CD8^+$) and SP ($CD4^+CD8^-$ and $CD4^-CD8^+$) T cells in the spleen of Adx rats (Fig 4B). In control Sham rats, the lymphocyte colonization of spleen did not occur, however, until day 21 of embryonic life. On the other hand, the thymus of 17-day-old fetal Adx rats contained, as those from 16-day-old Adx fetuses, significantly increased percentages of apoptotic cells (Table 1). We can, therefore, conclude that between days 16 and 17 of fetal life an important percentage of thymocytes undergoes in situ apoptosis or migrates from thymus to spleen in Adx rats. Remarkably, in this period, the absolute number of DN ($CD4^-CD8^-$) cells increased 10 times in Adx thymus, an event that was not found in the control thymus of any of the stages studied. This event was not correlated with an increase in the percentage of cycling DN cells compared with the values observed in Sham rats (data not shown), suggesting that a new wave of cell progenitors had colonized the thymus of 17-day-old Adx fetuses and were beginning to differentiate. In support of that, the percentage of DN cells was higher in Adx fetuses than in control ones and these later contained more $CD4^-CD8^+TcR\alpha\beta^-$cells (Fig 4A). Interestingly, in this stage, there was a significant reduction of the percentage of total cycling cells in Adx fetuses presumably due to the smaller proportion of immature $CD8^+$ cells, the thymocyte population that exhibits the highest proliferative index during ontogeny,[24,25] found in these animals.

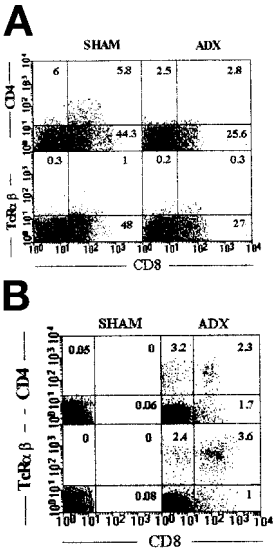

Fig 4. (A, B) Expression of CD4 (up) and TcRαβ (down) versus CD8 (horizontal axis) in thymocytes from 17-day old (A) and splenocytes from 18-day old (B), Sham (left) and Adx (right) fetuses. Results shown are representative of 3 to 4 experiments.

The recovery of control GC levels prevents the accelerated T cell maturation in Adx fetuses. To ensure that the observed changes in the T-cell development of Adx fetuses were caused by the absence of GCs rather than by the lack of other biological mediators produced by maternal adrenal glands, corticosterone replacement of Adx fetuses was performed using osmotic minipumps (2ML4, Alzet). After infusion of 23 µg/hour of corticosterone in a volume of 0.25 µL, the levels of hormone measured by RIA in the serum of 16- and 17-day-old fetuses were around 70 ng/mL. In these fetuses, both thymic cellularity and percentage of thymocyte populations (Fig 3C) defined by the CD4/CD8/TcRαβ expression were normal, demonstrating the relevance of the absence of circulating GC for the observed accelerated T-cell maturation in Adx fetuses.

The GC absence affects fetal liver thymic precursors. To investigate whether the acceleration of T-cell maturation in vivo observed in Adx fetuses was due to an effect of GC absence on the early progenitors, in vitro recolonization assays were performed. dGuo-pretreated thymic lobes from 15-day-old fetal mice were reconstituted with fetal liver cells either from 13-day-old Sham, and Adx fetuses. Previously, flow cytometry analysis showed similar numbers of $CD45^+(OX1^+)$ cells in the fetal liver of both groups of rats (data not shown). After 12 days of culture, more than 90% of yielded cells were rat thymocytes ($OX1^+$) without significant differences between the cultures supplied with fetal liver cells from control fetuses and those receiving fetal liver precursors from Adx fetal rats. However, there was a significantly higher proportion of $TcR\alpha\beta^{hi}$ cells

Table 2. Effect of the Lack of Maternal GCs in Recolonization Capacity of Fetal Liver Thymic Precursors

	TcRαβ^{hi}		% Mature Cells		Cycling Cells	
	%Total	%DP	CD4SP	CD8SP	%Total	%TcRαβ^{hi}
Sham	4.3 ± 0.7	3.3 ± 1.0	1.4 ± 0.3	0.6 ± 0.1	8.3 ± 1.3	15.1 ± 1.7
Adx	15.2 ± 2.1*	10.6 ± 1.8†	4.4 ± 0.8†	3.0 ± 0.6†	9.7 ± 2.4	14.2 ± 2.5

Phenotype and cell cycle analysis of thymocytes generated in dGuo-treated murine thymic lobes after 12 days of in vitro recolonization with fetal liver cells from either 13-day old control Sham or Adx rat fetuses. In both cases, the percentage of rat cells yielded was ≥90%, without significant differences between lobes reconstituted with control or Adx fetal liver cells. No cross-reaction for mouse thymic molecules of used MoAbs against rat molecules was observed. Data represent the average values of 3 to 4 experiments ± SD.

*$P \leq .01$.

†$P \leq .05$.

(around 3-fold) in the thymic lobes reconstituted with fetal liver Adx precursors (RL-Adx) than in those reconstituted with Sham ones (RL-Sham) (Table 2, Fig 5). Furthermore, this increased proportion of TcRαβ^{hi} cells found in the RL-Adx cultures included mature SP (both CD4 and CD8) cells, but also DP TcRαβ^{hi} thymocytes. To determine the origin of the increase of TcRαβ^{hi} thymocytes yielded in RL-Adx, we examined the proportion of cycling cells within this cell subset. Flow cytometry analysis of DNA cell content and TcRαβ expression showed that there were no significant differences between cycling cells among TcRαβ^{hi} thymocytes from RL-Adx and RL-Sham (Table 2). These results suggested that the higher numbers of mature thymocytes are not generated by expansion of preexisting cells, but presumably as a consequence of their accelerated production in the RL-Adx cultures.

In summary, the recolonization assays confirm a significant accelerated maturation of T-cell progenitors from Adx fetal liver, suggesting that T-cell precursors were affected by the absence of GC before their arrival at the early thymic primordium.

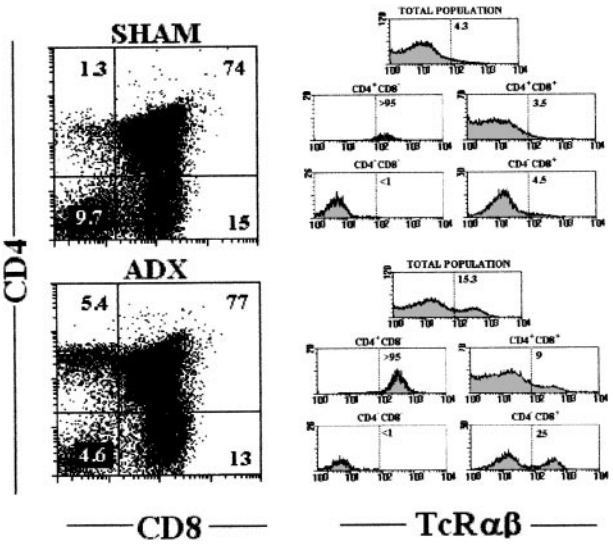

Fig 5. The GC absence affects fetal liver thymic precursors. CD4/CD8/TcRαβ expression in thymocytes generated in dGuo-treated murine thymic lobes after 12 days of in vitro recolonization with fetal liver cells from either 13-day-old control Sham (up) or Adx (down) rat fetuses. Histograms represent TcRαβ expression in the gated DN (CD4$^-$CD8$^-$), DP(CD4$^+$CD8$^+$), and SP (CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$) thymic cell populations shown in the dot plots. Results shown are a representative experiment with cell recoveries from RL-Sham of 85,000 cells/lobe and of 90,000 cells/lobe in the case of RL-Adx.

Early colonization of thymic primordium in Adx fetuses. As another possible cause accounting for the acceleration of T-cell maturation, the time of colonization of the early thymic primordium by lymphoid cells was examined comparatively in Adx and Sham fetuses. The ultrastructural results demonstrated that the thymic primordium of Adx fetuses was colonized earlier by lymphoid progenitors and developed faster than that of control, Sham fetal rats. At day 13 of gestation, the thymic primordium of control, Sham fetuses, consisted of homogeneous primitive epithelial cells, as previously reported,[24] many of which were in the process of division (Fig 6a), which began to establish a continuous supporting meshwork through incipient cell-to-cell contacts (Fig 6c), although wide intercellular spaces were still present (Fig 6a). On the contrary, the 13-day old Adx fetal thymus was largely invaded by lymphoid progenitors and contained numerous, more differentiated epithelial cells (Fig 6b). Invading lymphoid cells were round, electron-dense elements with patent nucleoli, a few round, electron lucent mitochondria, and numerous polyribosomes. Thymic epithelial cells were irregular elements joined together by incipient cell junctions (Fig 6d), which seemed to represent 2 distinct cell populations (Fig 6e). The most abundant epithelial cell type was an electron dense cell type, which contained long profiles of rough endoplasmic reticulum, numerous mitochondria, and occasional lipid droplets. In addition, a few irregular, electron lucent epithelial cells, containing a poor developed endoplasmic reticulum, an incipient Golgi complex, and some mitochondria occurred in the thymic primordium of 13-day-old Adx embryos.

DISCUSSION

GC function is mediated via specific receptors located in the cytoplasm of target cells. In the present work, we show that 13-day-old rat CD45$^+$ fetal liver cells, a population of cell progenitors that contains thymic precursors,[20-23] express GRs and that this expression is maintained in 15-day-old fetal thymocytes. In agreement, Ranelletti et al[26] demonstrated that human intrathymic precursors (CD3$^-$CD1$^-$ thymocytes) contain higher levels of GR sites per cell compared with the more mature CD3 positive population. More recently, an in situ hybridization study demonstrated that rat fetal liver contains GR mRNA as early as day 12 of fetal development when the hematopoietic activity of the organ begins.[2] Moreover, GR-specific gene expression was detected by these investigators in

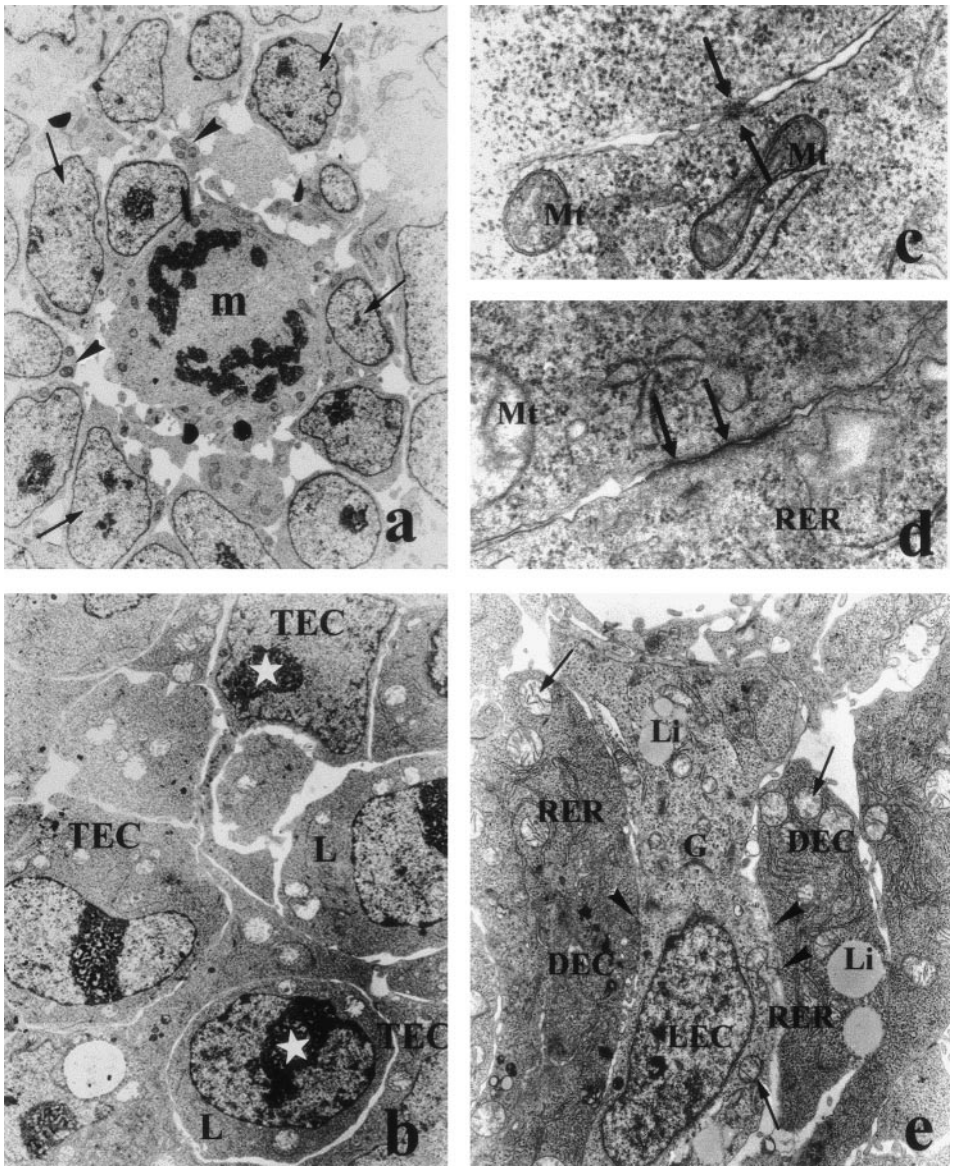

Fig 6. (a) Round or polygonal primitive epithelial cells (arrows), some of them in division (m), constitute the homogeneous thymic stroma devoid of lymphoid cells of a 13-day-old Sham fetal rat. Note the lack in these cells of prominent cytoplasmic organelles, except for a few, elongated mitochondria (arrow heads), and the enlarged intercellular spaces. Original magnification × 3,700. (b) Thymic primordium of a 13-day old Adx fetal rat. Numerous lymphoid progenitors (L) occur between a well constituted network of irregular thymic epithelial cells (TEC). Nucleolus (stars). Original magnification × 3,700. (c) Incipient cell-to-cell contact (arrows) between 2 primitive thymic epithelial cells of a 13-day-old Sham fetal rats. Mitochondria (Mt). Original magnification × 56,000. (d) Incipient cell junction (arrows) between 2 thymic epithelial cells of a 13-day-old Adx fetal thymus. Note the increased amount of cisternae of rough endoplasmic reticulum (RER) and mitochondria (Mt) as compared with the condition of Sham fetal rats of the same age (c). Original magnification × 35,700. (e) Electron-dense (DEC) and electron-lucent thymic epithelial cells (LEC), 13-day old Adx fetal thymus, rough endoplasmic reticulum (RER), lipid droplets (Li), mitochondria (arrows), Golgi complex (G), and incipient cell junctions (arrow heads). Original magnification × 6,000.

rat thymic primordium in 13-day-old fetuses, which increased on day 15. Although recently some studies have analyzed the effects of lack of GR signaling on thymus function,[1,7,15,27] these are largely devoted to the GC involvement in thymocyte selection and the physiologic relevance of GR expression in early thymic precursors has not been investigated.

The analysis of thymocyte ontogeny in the progeny of adrenalectomized pregnant rats allows an in vivo approach to this problem in which impairment of GR signaling is induced without blocking the expression of GR gene, but by eliminating the source of the hormone from the first day of fetal development. Vacchio et al[28] demonstrated that a fraction of mouse thymic epithelial cells could produce in vitro pregnelone and deoxicorticosterone that increases under the influence of adrenocorticotropic hormone (ACTH). Accordingly, this endogenous production could influence locally the thymocyte development after cell progenitors colonize the thymic primordia, but not before. Because an endogenouus production of GCs has not been demonstrated in fetal liver, where thymic cell progenitors are generated, we could conclude that maternal GCs are the only source of hormone for fetal liver cell progenitors until the establishment of the HPA axis[3,4] and its absence should be, therefore, responsible for the changes observed in the early development of thymic precursors of Adx fetuses. In fact, as shown in the current study, adrenalectomized pregnant rats provided with osmotic minipumps, which regularly release corticosterone, permit recovery of GC levels in early Adx fetuses and the normal development of thymocytes.

In mammalian fetuses, the thymic primordium is initially colonized by T-cell precursors from fetal liver, which rearrange the TcR genes to produce an initial repertoire that is rigorously selected resulting in T-cell effectiveness and tolerance.[29] In control rats, thymic primordium is colonized between 13 and 14 days of fetal life and the first DP ($CD4^+CD8^+$) cells appear on day 18. However, until day 20, mature T cells are not detected in the thymus and 1 day later in periphery.[24] In the progeny of Adx

rats, this chronology is, however, profoundly altered. As shown in our electron microscopy study, as early as day 13 of gestation, the thymic primordia is already colonized by lymphoid progenitors, and the thymic epithelial cells have begun to differentiate, whereas in control fetuses of the same age, an alymphoid thymic stroma consists of an homogeneous mass of primitive epithelial cells. This is followed by the appearance of DP thymocytes on day 15 of fetal life and 1 day later of mature SP, both CD4 and CD8, cells that colonize the spleen on day 18. Furthermore, in vitro recolonization assays demonstrate an altered behavior of thymic precursors occurring in the 13-day-old fetal liver which, together with the above-mentioned early colonization of thymic primordium, accounts for the faster development observed in the Adx fetuses. In agreement with these results, Castellanos et al[30] observed a faster recovery of thymic cellularity after irradiation in adrenalectomized compared with control adult rats.

On the other hand, the involvement of GCs in these processes is clearly demonstrated by the total recovery of normal T-cell differentiation in Adx fetuses in vivo receiving corticosterone. Moreover, after the establishment of fetal HPA axis, the thymocyte differentiation is progressively normalized, although the new wave of cell progenitors, which colonizes the thymus of Adx fetuses on day 17, exhibits accelerated maturation (Sacedón et al, manuscript in preparation).

As mentioned above, the role of GCs in the development of T-cell precursors has been little studied and the results obtained are incomplete and contradictory. Targeted disruption of the GR gene, which blocks the chromaffin tissue development and severely retards lung maturation, does not seem to affect, however, the thymus histology, although newborn and adult thymocytes are totally resistant to dexamethasone-induced apoptosis.[1] Recently, this same group has generated mice carrying a dimerization-defective GR.[7] The mutants lack inductibility of GC response elements (GRE) and show impairment of several important physiological functions, including GC-mediated thymocyte apoptosis. Surprisingly, despite this impairment of thymocyte apoptosis, a process associated with intrathymic T-cell selection, they were unable to detect any difference in the relative abundance of distinct thymocyte subsets defined by the CD4/CD8 cell profiles. Investigators recognize, however, that these are preliminary results, which need further confirmation, and in both studies, the ontogenetical development has not been analyzed. On the contrary, King et al[15] found that the partial blockage of GR expression in the thymus of transgenic mice expressing an antisense RNA for the GR under the control of lck proximal promoter, specifically expressed in thymus, triggered a drastic increase of apoptosis. This elevated cell death produced an important reduction of thymic cellularity from day 16 of fetal development, mainly due to a reduction of DP population, and a partial impairment of the normal progression of $CD4^-CD8^-$ cells to the $CD4^+CD8^+$ compartment. There are, however, important differences between this experimental model and that used in the current study. In the transgenic mice, the expression of antisense transcripts was specifically targeted at immature thymocytes using the lck proximal promoter. In this respect, during days 14 and 15 of fetal development, when early $CD3^-CD4^-CD8^-$ thymocyte precursors occupy the thymus, the cell recovery from control, nontransgenic and transgenic mice was remarkably identical.[15] On the contrary, our recolonization assays conclusively demonstrates that cell precursors are affected by the lack of circulating GCs before their arrival to the thymic primordium. In this regard, decreased numbers of the cell progenitors, which colonize the thymic anlagen, could be also contributing to the lower numbers of thymocytes observed in the Adx thymuses. Morale et al[27] explained, in agreement with the current results, changes in thymic cellularity and cell proliferation observed in the thymus of transgenic mice with a blockade of GR gene by alterations in the arrival of cell precursors into the thymus and/or their intrathymic proliferation.[27] Furthermore, the in vitro migration to the thymic supernatants of bone marrow cell progenitors obtained from dexamethasone or hydrocortisone long-term treated mice was significantly increased.[31] In both 16- and 17-day-old Adx thymuses, there are also increased percentages of apoptotic cells, which could account for reduced thymic cellularity observed in these animals. In agreement, King et al[15] reported an increase of apoptotic thymocytes in the absence of GCs. Nevertheless, because, as mentioned above, thymic epithelial cells are able to endogenously produce GCs,[28] it is possible to speculate that high levels of intrathymic cell death observed in the Adx fetuses could be due to an increased local production of GCs.

The present study provides, therefore, new information about the crucial role of GCs on T-cell differentiation. GCs not only regulate intrathymic T-cell maturation, but also exert a function on fetal liver cell precursors before thymic colonization and TcR$\alpha\beta$ expression.

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