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Epidermal Growth Factor (EGF) Modulates Fetal Thymocyte Growth and Differentiation: Partial Reversal by Insulin, Mimicking by Specific Inhibitors of EGF Receptor Tyrosine Kinase Activity, and Differential Expression of CD45 Phosphatase Isotypes

Claudia S. Freitas, Sergio R. Dalmau, and Wilson Savino

We have recently reported that epidermal growth factor (EGF) modulates thymocyte development in fetal thymus organ cultures. Exogenously added EGF arrested thymocyte growth and differentiation, acting at the transition from the CD4<sup>−</sup>CD8<sup>−</sup> (double-negative (DN)) to the CD4<sup>+</sup>CD8<sup>+</sup> (double-positive (DP)) phenotype. This inhibitory effect could be mimicked by tyrphostins, which are selective inhibitors of EGF receptor kinase activity. An attempt to use insulin (INS) as a synergizing effector resulted in partial restoration of lobe cellularity, leading to expression (2) and the down-regulation of CD25, which is the α-chain of the IL-2R (3), as well as of a very early thymocyte surface Ag, Fc<sub>RII/III</sub> (4, 5), are observed. This highly proliferative stage of thymocyte development involves signaling through p56<sup>ลก</sup>(3, 6), which is a nonreceptor-type protein tyrosine kinase (PTK) belonging to the src family that is expressed on thymocyte peripheral T lymphocyte membranes (7). This PTK is physically associated with the CD45 family of phosphatases (8–11), which is able to activate p56<sup>ลก</sup> through dephosphorylation of its inhibitory tyrosine residue 505 (12). Nevertheless, several aspects of thymocyte ontogeny with regard to cellular interactions, as well as signaling mechanisms during such critical stages preceding the TCR-mediated selective events, remain to be clarified.

Although both epidermal growth factor (EGF) receptors (EGFR) and EGF itself have been described in young thymuses (13, 14), evidence supporting an actual role for these molecules in thymus physiology is still scarce. We have recently reported that exogenous EGF promoted a dose-dependent modulation of thymocyte development in fetal thymus organ cultures (FTOCs). In its soluble form, this cytokine blocked thymocyte growth and differentiation, acting at the DN → DP transition (15). Such a blockade especially affected the TCR<sup>αβ</sup>− thymocyte subset, since TCR<sup>γδ</sup> (CD3<sup>+</sup>) thymocytes were found among the remaining EGF FTOC thymocytes, while TCR<sup>αβ</sup>− thymocytes were virtually absent (15). Interestingly, we detected by cytofluorometry an EGF-immunoreactive molecule on the surface of murine fetal and adult DN thymocytes whose expression was reduced in those cells remaining in a soluble EGF-treated FTOC (15).

A putative involvement of EGFR in the exogenous EGF-induced developmental blockade was suggested by the fact that TGF-α, which is another member of the EGF family that shares the same receptor (16), produced similar results in FTOCs. As an example, upon the addition of TGF-α (100 ng/ml), fetal thymus lobe cellularity was reduced from 390 × 10<sup>3</sup> in control FTOCs to 16 ×

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of these compounds display a highly selective inhibitory action, higher than the effective value for EGFR inhibition (18, 19). Some tyrosine residues as well as tyrosine phosphorylation of other molecules (17). Within this context, it appeared worthwhile to search for selective inhibitors of EGFR kinase activity, since nonspecific inhibitors might also affect other PTKs involved in thymocyte development, such as p56

Tyrophostins (TYRs) represent “tailor-made” PTK inhibitors designed to be used as antiproliferative or anticancer agents (18–21). Compounds of this group not only inhibit EGFR activity, but also other PTK-related activities, such as those triggered by platelet-derived growth factor receptor (22, 23), nerve growth factor receptor (24), and INS receptors (IR); however, in the latter case, the inhibitory concentrations used are three orders of magnitude higher than the effective value for EGFR inhibition (18, 19). Some of these compounds display a highly selective action, discriminating even between the highly homologous EGFR and ErbB2/ neu tyrosine kinase activities (22) or between p56

EGF and INS are growth factors used alone or in combination, often acting synergistically, in cultures of various cell types (27, 28). Both IR and EGFR are transmembrane PTKs that are able to autophosphorylate and initiate a phosphorylation cascade of intracellular messengers upon binding of their specific ligands (29–31). In the case of EGFR, members of the src family can be triggered (26, 32).

Taken together, these data prompted us to investigate the effect of INS on fetal thymocyte development within the context of an EGFR-driven blockade of FTOCs as well as a putative specific role for EGFR in this blockade. This approach revealed a condition in which growth and differentiation of the fetal thymocytes diverged, thus allowing a separate assessment of some molecular events involved.

Materials and Methods

Animals

C57BL/6J mice from the animal facilities of the National Cancer Institute of Rio de Janeiro were used. Females were bred overnight, separated from males in the morning (day 0), and maintained on a diet supplemented with sunflower and corn seeds for 14 days. Pregnant females were killed by ether anesthesia, and fetuses were harvested under sterile conditions.

Cytokines

Natural murine EGF (mEGF) from mouse submaxillary glands was obtained from Sigma (St. Louis, MO). Purified porcine and bovine INS (Central de Medicamentos do Ministério da Saúde, Rio de Janeiro, Brazil) and human recombinant INS (Sigma) were used, and their activities did not differ qualitatively or quantitatively. The other cytokines used in the present study were human rIL-2 (Hoffman-La Roche, Nutley, NJ; kindly provided by Dr. Richard Peck, Basel Institute for Immunology, Basel, Switzerland), murine rIL-7 (R&D Systems, Minneapolis, MN), and human rTGF-α (Life Technologies, Gaithersburg, MD).

TYRs

TYRs 1 (IC50 of >1250 μM), 25 (IC50 = 3 μM), and 51 (IC50 = 0.8 μM) were purchased from Sigma. The IC50 refers to the inhibitory effect observed for these compounds on EGFR autophosphorylation in acellular systems (19). TYR AG1478 and the p56

Rat anti-mouse CD4/phycoerythrin (PE) (clone GK1.5) and CD8/FITC (clone 53-6.7) mAbs as well as the PE-coupled streptavidin and the irrelevant mouse keyhole limpet hemocyanin FITC and PE-labeled Ab were obtained from Becton Dickinson (San Jose, CA). Rat anti-mouse CD25/FITC (clone AMT13), CD4/quantum red (QR) (clone H29.19), CD8a/QR (clone 53-6.7), and CD44/QR (clone IM7.8.1) mAbs as well as rabbit anti-mEGF polyclonal antisera and goat anti-rabbit IgG/FITC secondary Ab were obtained from Sigma. Rat anti-mouse CD45RB/PE and CD45RC/PE (clones 23G2 and DNL-1.9, respectively), CD4/FITC (clone H129.19), and CD2/biotin (clone RM2-5) Abs as well as hamster anti-mouse TCRα/β/PE (clone GL3) and anti-mouse CD3ε/biotin (clone 145-2C11) were purchased from PharMingen (San Diego, CA), whereas hamster anti-mouse CD3ε/FITC (clone 145-2C11) was purchased from Boehringer Mannheim (Mannheim, Germany). Rat anti-mouse TCRβ/QR- biotin (clone H57-597) and goat anti-rat IgG/PE secondary Ab were obtained from Life Technologies. Streptavidin-tricolor was purchased from Caltag (San Francisco, CA), and anti-mouse FcγRII/III (clone 2.4G2) was an ascites that was kindly provided by Dr. Mireille Dardenne (Hôpital Necker, Paris, France). The nonstained rat Igs were prepared in our laboratory from normal rat serum by ammonium sulfate precipitation.

FTOCs

FTOCs were performed as described in the literature (33), with some modifications. Using watchmaker forceps, 14- to 15-day-old fetal thymuses were excised and assembled (5–10 per dish) on a 0.22-μm Millipore membrane (Bedford, MA) supported by a stainless steel grid inside a Nunc delta-plate (Nunclon, Roskilde, Denmark) containing 1 ml of culture medium (DMEM 310 mOsm) plus 10% FCS (defined serum, HyClone, Logan, Utah), glutamine, nonessential amino acids (Life Technologies), 60 mg/L penicillin, and 100 mg/L streptomycin. Complete medium was changed every 3 days; after 7 to 10 days of culture, lobes were harvested into a small volume of medium and smashed under a glass coverslip. Thymocytes were recovered, counted, and then suspended in ice-cold medium for further flow cytometry processing. When working with TYRs, culture media were changed every day due to their rapid degradation (20).

Radioactive thymidine incorporation

Pooled thymus lobes were initially incubated for 40 h with the growth factors. Cells were subsequently recovered, and 1.5 × 106 thymocytes (200 μl in 96-well plates) from each pool were further incubated with 3 μCi of [3H]Tdr (specific activity of 85 Ci/mM) for 4 h. Measurements of radioactive incorporation were run in triplicate. Statistical analysis was performed by the Student t test using the SigmaStat computer program (Sigma).

Flow cytometry

Cells maintained in ice-cold medium or HBSS plus 2% FCS were submitted to double or triple immunostaining. Labelings with irrelevant Abs were run in parallel to set a negative threshold; in some cases, unstained normal rat Igs were used as blocking agents to preincubate the cells. To exclude dead cells, thymocyte suspensions were further treated with propidium iodide (PI) at a final concentration of 2 μg/ml. The material was analyzed using a FACScan (Becton Dickinson) equipped with a 15-mW air-cooled 488-nm argon ion laser. Appropriate electronic compensation was applied between the fluorescence channels to remove spectral overlapping. Data acquisition was conducted using the Lysis II software (Becton Dickinson); 20,000 to 50,000 events were recorded from each sample.

For cell cycle determination, nuclei were stained for 15 min at room temperature with 50 μg/ml PI in a 4-nM trisodium citrate solution containing 0.3% Triton X-100, and the RNA was digested for an additional 15 min with 100 μg/ml of bovine pancreatic RNase (5–10 Kunitz U/ml; Sigma) in 40 mM trisodium citrate (pH 8.2). Cell cycle phase was determined using the CellFit program and the SIFT or RFTT mathematical model.

Results

EGF-driven down-modulation of thymocyte growth in FTOCs: partial reversal by INS

The previously reported blockade of fetal thymus development upon the addition of exogenous EGF (15) was approached herein using suboptimal EGF doses plus INS. The latter was applied to FTOCs at 20 to 40 nM doses, which is compatible with its use as

102 in TGF-α-treated FTOCs or to 66 × 103 in TGF-α+insulin (INS)-treated FTOCs. However, it is not known whether the addition of such growth factors can affect thymus development in culture through EGFR-dependent signaling pathways or through internalization and down-regulation of the EGFR, leading to a blockade of its signaling activity, which includes autophosphorylation of tyrosine residues as well as tyrosine phosphorylation of other molecules (17). Within this context, it appeared worthwhile to search for selective inhibitors of EGFR kinase activity, since nonspecific inhibitors might also affect other PTKs involved in thymocyte development, such as p56

Taken together, these data prompted us to investigate the effect of INS on fetal thymocyte development within the context of an EGFR-driven blockade of FTOCs as well as a putative specific role for EGFR in this blockade. This approach revealed a condition in which growth and differentiation of the fetal thymocytes diverged, thus allowing a separate assessment of some molecular events involved.

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a synergistic effector of the EGF growth-inducing action as reported for cultures of various cell types (34). Unexpectedly, instead of cooperating with EGF, INS could partially, yet consistently, reverse the reduction in the total numbers of thymocytes grown in 7-day FTOCs, even in the presence of optimal doses of exogenous EGF (Fig. 1). INS promoted an increase in both thymocyte numbers (Fig. 1) and in the size of thymus lobes (data not shown) both in the presence and absence of EGF, indicating a growth-inducing activity for the hormone itself in this culture system. In fact, in short-term cultures, INS could not counteract the early decrease in thymocyte cycling activity imposed by EGF addition; this finding argues against a direct interference with a putative EGF-induced toxicity (Table I). In full-term (7-day) cultures, this growth-inducing activity was expressed as a preferential expansion of DN thymocytes (Fig. 2). EGF was used herein at 10 ng/ml, which is an optimal dose for blocking thymocyte growth but is suboptimal for differentiation. When cells from such cultures at suboptimal EGF doses were triple-stained with the anti-CD3ε, anti-CD25, and anti-CD44 mAbs, a change in the labeling pattern of the immature thymocyte subsets (mostly CD3−) was seen, with INS enhancing the growth of cells bearing the CD3− CD44− CD25+ phenotype when compared with FTOCs treated with EGF alone (Fig. 3). The expanded CD44− subset contained mainly DN cells, as ascertained by triple CD4, CD8, and CD44 staining (data not shown). Additionally, these expanding cells expressed high levels of c-kit (data not shown). INS addition to FTOCs containing higher EGF doses such as 100 ng/ml which are optimal for blocking both thymocyte growth and differentiation in FTOCs also induced an expansion of the DN CD44− CD25− subset (data not shown). Furthermore, when thymocytes from EGF- and EGF+INS-treated FTOCs were assessed for other events that characterize the DN → DP transition, such as loss of FcγRII/III and acquisition of CD2, we could observe a preponderant FcγRII/III CD2+ phenotype (Fig. 4). Similar to what occurred with the DN cells from EGF-treated FTOCs (15), those from EGF+INS-treated FTOCs showed reduced labeling with rabbit anti-mEGF polyclonal antisera compared with the DN cells from untreated and INS-treated FTOCs, in which a substantial percentage was positively labeled by this antiserum (Fig. 5A). Also similar was the preferential blockade of the development of the TCRαβ+ thymocyte subset compared with that of the TCRγδ− subset, since TCRαβ+ cells were virtually absent in both EGF- and EGF+INS-treated FTOCs (Fig. 6). This finding is not surprising, since TCRγδ+ cell development differs from that of their TCRαβ− counterparts in many aspects; for example, TCRγδ− cells are not subjected to TCRβ selection in fetal life (35) and do not require p56lck function to develop in the thymus (6).

**Mimicking of the action of soluble EGF by TYRs**

When TYRs were added to 14-day fetal thymus lobes grown for 7 days in FTOCs, a blockade of thymocyte growth and differentiation was imposed by TYRs 25 and 51 (Fig. 7) and by the highly

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**FIGURE 1.** EGF-driven down-regulation of thymocyte growth in FTOCs: partial reversal by INS. Fetal thymus lobes were incubated for 7 to 10 days in the presence of different EGF concentrations (nanograms per milliliter) with or without INS. In each experiment, the mean cell number per lobe was calculated from pools of 5 to 10 lobes per point. Based on these control values, which were taken as 100%, the percentage of modulation was calculated for each growth factor-containing point. Mean ± SEM of percentages from a series of experiments is shown. Values in parentheses represent the number of experiments performed for each particular point. Absolute cell numbers in control FTOCs varied from ~2 to 5 x 10⁶ per lobe. INS was used at 20 to 40 nM.

**FIGURE 2.** Exogenous INS stimulates the growth of DN thymocytes in FTOCs. Cytocfluorometric profiles of CD4 and CD8 double-labeled fetal thymocytes after a 7-day FTOC in the presence or absence of growth factors are shown from one representative experiment of four. Values within each panel represent percentages for each cell subset; cellularity per lobe (x10^−³), which is the mean from a pool of lobes, is seen at bottom right. Fluorescence intensity is plotted in log scale. EGF was added at 10 ng/ml (~1.6 nM); INS was added at 40 nM.

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Table I. Early effects of EGF and INS on fetal thymocyte proliferation in FTOCs.

<table>
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<th>G1%</th>
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<tr>
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<td>11.037 ± 4.596</td>
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<td>EGF+INS</td>
<td>81.1 ± 7.0</td>
<td>19.1 ± 6.8</td>
<td>3.956 ± 912</td>
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</tbody>
</table>

*Fetal thymus lobes were cultured for 24 h in the presence or absence of 100 ng/ml of EGF (~16 nM) and 40 nM of INS, pooled, and smashed in 96-well plates with 3μCi [3H]Tdr. Data are reported as means ± SEM.

**Proliferation** was also ascertained by radioactive thymidine uptake. Fetal thymus lobes were cultured for 40 h with or without growth factors, harvested, and smashed; the thymocytes were counted and incubated (1.5 x 10⁶ cells/well) for an additional 4 h in 96-well plates with 3μCi [3H]Tdr. Data are reported as means ± SEM (cpm) of three experiments in triplicate.

**Mean values are significantly different from control FTOC values as assessed by S+G2+M determination (p = 0.001) or by [3H]Tdr incorporation (p = 0.02).**

**Mean values are not significantly different from EGF FTOC values as assessed by S+G2+M determination (p = 0.06) or by [3H]Tdr incorporation (p = 0.02).**

**Mean values are not significantly different from EGF FTOC values as assessed by S+G2+M determination (p = 0.72) or by [3H]Tdr incorporation (p = 0.37).**
selective inhibitor of EGFR PTK activity, TYR AG1478 (Fig. 8).

TYR 1, used herein as a control due to its high IC₅₀, did not substantially modify the cellularity or the CD4/CD8-defined thymocyte differentiation profile observed in cultures to which DMSO was added as a control for the solvent (Fig. 7). Again, in analogy to what we observed regarding the EGF-related dose-response curve (15), thymocyte growth was first sensitive to blockade at a lower TYR dose as compared with differentiation, which was affected when the dose was raised (Fig. 8). Also analogous is the fact that most of the DN thymocytes obtained from AG1478 FTOCs were negative for CD2 (Fig. 9A), either CD44⁺CD25⁻ or CD44⁺CD25⁺ (Fig. 9B), and for anti-EGF staining (Fig. 5B). Interestingly, TYRs 51 and AG1478 could not prevent the growth-inducing activity of INS (Figs. 7–9), as was also observed in PP2 FTOCs (Fig. 9, A and B). Even in the presence of these compounds, INS favored the expansion of the CD44⁺CD25⁻ subset in FTOCs (Fig. 9B).

**CD45RB phosphatase isotype expression precedes the DN → DP transition**

As shown in Figure 10A, the early thymocyte cycle arrest in response to 100 ng/ml of exogenous EGF (Table I) was accompanied by an arrest in the DN → DP transition in 15.5-day fetal thymus lobes incubated in FTOCs overnight. The dual-color staining of these cells with anti-CD4/FITC and anti-CD8/FITC plus PE-coupled anti-CD45RC or anti-CD45RB mAb revealed that the membrane expression of one particular CD45 isotype (CD45RB but not CD45RC) was enhanced to high levels before the DN → DP transition (Fig. 10A). Interestingly, such an expression of the CD45RB isotype was observed to occur in vivo as well, as revealed by cytofluorometric analysis of noncultured thymocytes from 15- to 17-day-old fetuses (Fig. 10B).

In thymus organ cultures, the positive CD45RB staining was maintained after the acquisition of the DP phenotype, as observed 1 wk later in both untreated and INS-treated FTOCs (Fig. 10C). However, in the presence of EGF, only a small subset of cells evolved to the CD45RB⁺ phenotype; the majority of cells were retained as DN CD45RB⁻ thymocytes (Fig. 10C). This CD45RB⁻ profile persisted when INS was added, revealing that expression of the CD45RB isotype is not related to the immature thymocyte expansion at this stage, but rather to subsequent events.

The results obtained by the use of diverse TYRs, whose inhibitory effects upon FTOCs gradually vary, counteracting the growth-inducing activity found for the solvent DMSO on immature thymocytes (Figs. 7 and 8), further illustrate that the CD45RB acquisition precedes the appearance of the CD4/CD8 DN phenotype in fetal thymocyte development (Fig. 11). Again, as ascertained by this parameter, these compounds mimicked the EGF-driven effects.

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**FIGURE 3.** INS addition to FTOCs favors the growth of CD3⁻ cells expressing a CD44⁺CD25⁺ phenotype. Fetal thymocytes from a 7-day FTOC in the presence or absence of growth factors were triple-stained with anti-CD25, anti-CD44, and anti-CD3ε. The plots in R1, R2, R3, and R4 represent analyses of CD44/CD25 patterns for regions from anti-CD3ε histograms: R1 represents the first region at left (negative). Values within histograms represent cellularity per lobe (i.e., the mean from a pool of lobes) (×10⁻³). Within each panel of two-dimensional plots, numbers represent percentages from total living cells distributed among the CD3 subsets. Fluorescence intensity is plotted in log scale. EGF was added at 10 ng/ml, and INS was added at 40 nM. Similar results were observed in two additional experiments with EGF at 10 ng/ml as well as in two additional experiments using EGF at a 10 times higher dose (100 ng/ml).

**FIGURE 4.** EGF blocks thymocyte development at the FcγRII/III "CD2⁻" stage. A typical experiment is shown in which thymocytes from pooled lobes were preincubated with nonstained normal rat Igs and then incubated with rat anti-FcγRII/III mAb followed by goat anti-rat PE serum. Cells were subjected to nonstained rat Igs again and subsequently to rat anti-mouse CD4- and anti-CD8/FITC-conjugated mAbs plus rat anti-mouse CD2/biotin followed by streptavidin-tricolor. Values within each panel represent percentages for each cell subset. Fluorescence intensity is plotted in log scale. INS addition to FTOCs did not change the EGF-driven impairment of FcγRII/III loss and CD2 acquisition.
Discussion

In the present study, experiments were designed to better understand the molecular basis related to our recently reported EGF-driven blockade of thymocyte growth and differentiation in the murine FTOC model.

The addition of INS to FTOCs at doses compatible with its use as a synergistic effector for the EGF growth-inducing action in a number of cell types (20–40 nM) (34) partially reversed the EGF-induced effects with regard to the blockade of thymocyte growth. In addition, a sustained growth-inducing activity was evidenced for INS itself from 10 to 80 nM (160% recovery when control FTOCs were taken as 100%; 135% at the 160-nM dose). However, even at the highest doses tested (80 and 160 nM; data not shown), INS was not able to reverse the EGF-induced blockade of thymocyte differentiation from the DN to the DP phenotype, shown here using 20 to 40 nM doses. These nanomolar doses contrast with the picomolar maximal concentrations found physiologically in blood. However, since INS expression was recently demonstrated in human and mouse thymus (36–38), an autocrine/paracrine role of this hormone in early thymocyte development is conceivable. This possibility is supported by the presence of IR in both thymic epithelial cells and thymocytes from fetal murine and human thymuses (39). Accordingly, we may propose a direct action of INS on immature thymocytes or an indirect effect mediated by IR-bearing microenvironmental cells (for example, through the release of other growth-inducing cytokines). In this respect, INS was found to enhance the production of IL-1α, IL-6, and granulocyte-macrophage CSF by human thymus epithelial cells (40). Alternatively, exogenous INS may evidence the action of other cytokines putatively present in the thymic microenvironment, potentiating their intracellular signal transduction pathways. In fact, intracellular INS substrates such as FIGURE 5. Down-regulation of immunoreactive EGF on the surface of DN thymocytes. A, Pooled lobes from untreated, INS-treated, and EGF+INS-treated FTOCs were double-labeled with anti-CD4- and anti-CD8/QR-conjugated mAbs plus rabbit anti-mouse EGF polyclonal antiserum followed by goat anti-rabbit FITC serum. Labeling controls received the secondary Ab alone. Values within each panel represent percentages for each cell subset. Fluorescence intensity is plotted in log scale. Note that a substantial part of the DN thymocytes from either the untreated or INS-treated FTOCs show anti-EGF reactivity, whereas most cells in the EGF+INS-treated FTOCs are anti-EGF−. B, Pooled lobes from untreated, DMSO-, AG1478-, and AG1478+INS-treated cultures were stained as described above. The final concentration was 40 µM for TYR, 40 nM for INS, and 0.4% for DMSO.

FIGURE 6. Recovery from FTOCs of CD3+ thymocytes bearing TCRαβ or TCRγδ. A typical experiment is shown in which fetal thymocytes from pooled lobes were harvested from FTOCs with or without growth factors and stained with anti-CD3ε mAbs plus either anti-mouse TCRγδ or anti-mouse TCRαβ. Cells were stained with irrelevant Abs as a control for nonspecific labeling and autofluorescent signals and were used to set the positivity threshold. Fluorescence intensity is plotted in log scale. Values within each panel represent percentages of CD3+ TCR+ cells. Development of the TCRαβ+ subset was preferentially affected by EGF addition.
IR substrate-1 may participate in a cascade of transacting signals, such as those displayed by Jak-2 under the stimulus of growth hormone, IFN-γ, or leukemia inhibitory factor in other cell culture systems (41), as long as the activity of these kinases is not affected by the TYRs used here (not assayed). Other factors assayed in FTOCs were IFN-γ and INS-like growth factors I and II, which did not elicit significant thymocyte growth-inducing activity in our system (data not shown).

It is important to mention that in the present study, although favoring the growth of an immature thymocyte subset in FTOCs, INS could not act as a fetal thymocyte growth factor when the lobe architecture was disrupted in lobe submersion cultures, in contrast to the actions of IL-7 and IL-2, which reportedly expand immature thymocyte subsets under the same conditions (42). INS cannot substitute for those factors in lobe submersion cultures or maintain the growth of IL-7-expanded fetal thymocytes in suspension in the absence of stromal components (data not shown).

Both INS and members of its family reportedly act positively on thymus cellularity (43) in addition to counteracting thymus atrophy in diabetic rats (44) and aiding in the recovery of cyclosporin A-atrophied thymus (45). In our experiments in long-term FTOCs in which 14-day fetal lobes were submitted to a 1-wk incubation with EGF (100 ng/ml) and then allowed to recover for 10 days in the absence of this factor, the treatment with 20 nM of INS during recovery increased the final cellularity from $1.95 \times 10^5$ in control lobes (in the absence of INS) to $3.9 \times 10^5$ per lobe, a rescuing activity resembling that reported in previous studies.

A second point is the involvement of EGFR in the EGF-driven blockade of thymocyte growth and differentiation in the murine FTOC model, suggested by the fact that TGF-α, another member of the EGF family that shares the same receptor (16), produced similar results. We approached this point using TYRs 25 and 51, which are known to block EGFR PTK activity at a low IC50 compared with TYR 1, which shows a high IC50 and was used as a control. TYR AG1478 was also used because, in addition to being more potent, it is known as a highly selective EGFR inhibitor (23, 26). According to the literature, TYRs do not alter the cell surface display of EGFR, EGF binding, or EGF-induced internalization, degradation, and down-regulation of the receptor (30); however, TYRs do prevent the latter from signaling through tyrosine phosphorylation of substrates (19). In the particular case of AG1478, this receptor was sequestered in the form of inactive homodimers, apparently due to the ability of the quinazoline to interact with ATP binding sites in EGFR (46).

In our experiments, the addition of TYRs to EGF FTOCs did not reverse the EGF-induced blockade of lobe development (data not shown), which argues against an EGFR PTK-elicited cascade of intracellular inhibitory signals (47). Instead, TYRs 25, 51, and AG1478 themselves produced in FTOCs a blockade of thymocyte growth and development analogous to that imposed by exogenous EGF addition, suggesting that the soluble EGF blocking action is related to receptor unavailability rather than to its signaling properties. This possibility would reinforce our hypothesis that soluble EGF cooperate with the growth factor activity of INS to generate the milieu favoring the growth of immature thymocytes in FTOCs.
EGF acts by preventing an endogenous signal from being delivered, which could involve the membrane-anchored EGF-immuno-reactive 120-kDa polypeptide seen in fetal thymocytes (15) or even other immobilized molecules containing EGF motifs. In fact, a large family of secreted or cell surface-anchored proteins can be defined by the presence of EGF-like repeats; their role in cell-to-cell interactions was shown to be important in development as adhesion mediators and possibly also as EGFR activators (48).

Although it is appealing to assume that the TYRs used here operated via EGFR, due to the apparent relationship between the IC₅₀ for this receptor inhibition and the efficiency of the DN → DP blockade, it remains possible that, in addition to EGFR, other undefined PTK targets are involved in TYR blockade, especially in the case of the less selective compounds (TYRs 25 and 51). Even in the case of AG1478, which is known for its highly selective action, other targets may be proposed. Among them are members of the diverse tyrosine kinase families expressed in leukocytes (49), including those from the src family to which p56lk belongs.

This last kinase is known to elicit signals that are critical precisely at this transition (3, 6). In this respect, although AG1478 is reportedly ineffective against the immunopurified kinases of the src family to which p56lk belongs, its action was not tested against this

![FIGURE 9. Thymocyte surface phenotype under the action of the EGFR inhibitor AG1478 or the p56lk inhibitor PP2. Thymus lobes were cultured for 7 days in the presence of these compounds (with or without INS), pooled, and smashed; the thymocytes were stained as described previously. Values within panels represent percentages for each cell subset; cellularity per lobe (×10⁻³), which is seen at bottom right, corresponds to the mean values from pools of 6 to 12 lobes. Upon the addition of AG1478 or PP2, a retention of thymocytes mainly at the DN CD2⁻ developmental stage (either CD44⁺CD25⁻ or CD44⁻CD25⁺) was observed. The last subset preferentially expanded under the action of INS. AG1478 was applied at 40 μM; PP2 was applied at 2.5 μM in A and at 4 μM in B.](http://www.jimmunol.org/)

![FIGURE 10. CD45RB but not CD45RC isotype expression is increased before the DN → DP phenotype transition. A, Thymus lobes from 15.5 fetuses were cultured overnight with or without EGF. Cells harvested from pooled lobes were double-labeled with anti-CD4- and anti-CD8/FITC-conjugated mAbs plus anti-CD45RB or anti-CD45RC/PE-conjugated mAbs before cytofluorometric analysis. Other pools received PE- and FITC-coupled irrelevant Abs to set threshold-positive labeling. Fluorescence intensity is plotted in log scale. Values within each panel represent percentages for each cell subset from one of two experiments. B, Fetal thymuses were excised, and thymocytes were harvested and directly stained for analysis. For each pool of cells, a nonstained sample was used to set threshold-positive labeling. Other pools received PE- and FITC-coupled irrelevant Abs to set threshold-positive labeling. Fluorescence intensity is plotted in log scale. Values within each panel represent percentages for each cell subset from one of two experiments. C, Fetal thymus lobes were cultured for 7 days with or without growth factors. Thymocytes from pooled lobes were harvested and cytofluorometrically analyzed as described above.](http://www.jimmunol.org/)
particular enzyme. Other putative targets are, for example, members of the Jak kinase family, which were shown to act at a similarly early stage of T cell development, with mutations in Jak-3 resulting in SCID disease (49). Inhibition of the activity of members of the Jak kinase family is likely to block cellular responses to multiple cytokines and growth factors (49). However, these putative additional effects of AG1478 did not affect the exogenous EGF-induced blockade (data not shown) or the INS-induced growth of immature thymocyte subsets. It is possible that the resistance of INS action to TYRs 51 and AG1478 is related to the requirement of a much higher dose of EGFR blockers to inhibit the IR-associated PTK activity (19) or alternatively to non-PTK modes of INS action, among the pleiotropic effects displayed by this hormone (21). The thymocyte growth-inducing activity of INS was shown herein to occur independently of p56

According to the literature, cycling thymocytes at the CD44+CD25+ developmental stage, such as those expanding upon INS action, are thought to represent either the remaining cycling cells from the preceding CD44+CD25+ subset or the β-selected population expanding upon pre-TCR signaling (50, 51). CD25 down-regulation seems to be at the frontier between these two proliferative stages, with the second being strictly p56

It was shown that the persistence of TCR genes in germline configuration has a dramatic effect on CD2 expression (5), and that transfection of a TCRβ chain gene into a SCID-derived cell line leads to up-regulation of CD2 surface expression in addition to a TCRβ-β homodimer (52). Our findings concerning both EGF FTOCs and EGF+INS FTOCs suggest that soluble EGF blocked thymus development before the TCRβ chain rearrangement and/or expression, since thymocytes did not reach the FcγRII/IIIa-CD2

FIGURE 11. TYRs affect CD45RB expression at the DN → DP transition. Fetal thymus lobes were cultured for 7 days in the presence of TYRs 1 (control TYR), 51, or AG1478, or with DMSO (used as a control for the solvent). The medium with or without newly added TYRs was changed every day. Thymocytes were obtained from pooled lobes and double-labeled with anti-CD4- and anti-CD8/FITC-conjugated mAbs, plus anti-CD45RB/PE mAbs. Fluorescence intensity is plotted in log scale. Values within each panel represent percentages for each cell subset. The progressive blockade in the acquisition the DP phenotype further illustrates that CD45RB acquisition precedes the EGFR-mediated point of arrest.

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References


