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SUBPOPULATIONS OF FETAL THYMOCYTES DEFINED BY EXPRESSION OF T CELL RECEPTOR/CD3 AND IL-2 RECEPTOR

CD3 and IL-2 Receptor α-Chain Are Expressed on Reciprocal Cell Populations

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The expression of the TCR/CD3 complex and the IL-2Rα chain (p55) on fetal thymocytes has been analyzed by flow cytometry (FCM). Two-parameter immunofluorescence identified three subpopulations which were respectively IL-2Rα$^-$/CD3$^+$, IL-2Rα$^+$/CD3$^-$, or IL-2Rα$^-$/CD3$^-$; no detectable population of IL-2Rα$^+$/CD3$^+$ cells was found in unstimulated fetal thymocytes. Fractionation by "panning" and by sterile flow cytometric separation was used to characterize the functional responsiveness of these three subpopulations to a variety of stimuli. All three populations proliferated in response to PMA + ionomycin + rIL-2. In contrast, stimulation with anti-CD3 + IL-2 induced proliferation in IL-2Rα$^-$/CD3$^+$ and IL-2Rα$^-$/CD3$^-$ but not in IL-2Rα$^+$/CD3$^-$ thymocytes. IL-2Rα$^-$ cells, including sorted IL-2Rα$^-$/CD3$^-$ thymocytes, underwent a phenotypic change in response to in vitro stimulation with anti-CD3 + IL-2, resulting in the appearance of an IL-2Rα$^+$/CD3$^+$ population that was not detected in freshly isolated thymocytes. The ability of fractionated fetal thymocytes to produce lymphokine in response to PMA + ionomycin was also evaluated. Only the IL-2Rα$^-$/CD3$^-$ fraction generated detectable IL-2. These findings demonstrate for the first time that CD3 and IL-2Rα are expressed in a mutually exclusive fashion in fetal thymocytes and define three subpopulations of thymocytes that differ significantly in their proliferative and differentiative responses to TCR-mediated, IL-2R-mediated, and pharmacologic stimulation.

During fetal development, thymocytes undergo a highly programed sequence of differentiation steps that results in the generation of mature Ag-specific T cells that are then exported to the peripheral lymphoid population (1). This maturation process is reflected in the ordered expression of a number of cell surface molecules that serve as markers for functionally distinct cell populations (1). The process of T cell maturation presumably involves interactions at several points between receptor structures expressed on developing T cells and ligands present in the thymic maturation environment. Among the cell surface receptors that are known to play critical roles in the activation and function of mature T lymphocytes are the TCR, and the IL-2R. In their resting state, most mature T cells constitutively express cell surface TCR. In contrast, expression of the IL-2R α-chain generally depends on delivery of an activating signal to the T cell, a signal that is often delivered through the TCR. The induced expression of IL-2R renders T cells responsive to IL-2, leading to T cell proliferation and functional differentiation. However, the relative roles of the TCR and IL-2R during fetal T cell development are less clear. Expression of the TCR is a well established event during intrathymic T cell development and is critical for both positive and negative repertoire selection (1). Expression of the IL-2R precedes that of the TCR in thymic development, but its functional significance is controversial. Conflicting reports have concluded that immature thymocytes are either able or unable to respond to IL-2 in vitro (2–10). Similarly, organ culture and in vivo studies have concluded that antibodies specific for the IL-2R either do or do not influence thymic differentiation (11–13). It is also unclear what stimuli are responsible for expression of IL-2R by fetal thymocytes at various stages of development.

The present study has analyzed expression of the TCR/CD3 complex and the IL-2Rα chain (p55) in the fetal thymus by FCM[3]. Three subpopulations were identified by two-parameter immunofluorescence that were respectively IL-2Rα$^-$/CD3$^+$, IL-2Rα$^+$/CD3$^-$, or IL-2Rα$^-$/CD3$^-$; no detectable population of IL-2Rα$^+$/CD3$^-$ cells was found in unstimulated fetal thymocytes. These three subpopulations of fetal thymocytes were found to differ significantly in their proliferative and differentiative responses to TCR-mediated, IL-2R-mediated, and pharmacologic stimulation.

MATERIALS AND METHODS

Mice. Timed pregnant (BALB/c × DBA/2)F$_1$ (CD2F1) mice were obtained from the Frederick Cancer Research Facility, Frederick, MD.

Reagents. 7D4 (14), a rat mAb reactive to the p55 (α) chain of the mouse IL-2R, was a gift of E. Shevach (NIH). 145-2C11 (15), a hamster mAb specific for the ε-chain of the mouse CD3 complex, was a gift of J. Bluestone (University of Chicago, Chicago, IL). Conjugates of anti-CD4 (H129.19) (16) were generously provided by J. Titus (NIH). 11B11 (17), a mAb specific for mouse IL-4, was a gift of W. Paul (NIH). S4B6 (18), a mAb specific for mouse IL-2, was a gift

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[3] Abbreviation used in this paper: FCM, flow cytometry.

of T. Mosmann (DNAX). Anti-FcR mAb 2.4G2 (19) was provided by J. Titus (NIH). Biotin- or fluorescein-conjugated mAb specific for Thy-1.2, Ly-1 (CD5), Lyt-2 (CD8), and human CD3 (Leu 4) were purchased from Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA. rIL-2 was provided by Cetus Corp. (Emeryville, CA). rIL-4 was provided by W. Paul and by S. Gillis (Immunex). PMA was purchased from Sigma Chemical Co. (St. Louis, MO) and ionomycin was purchased from Calbiochem (La Jolla, CA).

Cell preparation and fractionation. Thymus lobes from embryos of known gestational age were removed under a dissecting microscope and mechanically disrupted to make single cell suspensions. Fractionation was carried out by incubating cells for 1 h at room temperature on tissue culture dishes (Nunc, Denmark) that had been coated with 10 μg/ml 7D4 in borate buffer (pH 8.5). At the end of this incubation period, non-adherent cells (an average yield of 70.3%) were removed by gentle agitation and were then treated with 7D4 mAb and rabbit C′ (Cedarlane, Hornby, Ontario, Canada) to eliminate residual $7D4^+$ cells; the resulting $7D4^-$ population contained both $7D4^-/2C11^+$ and $7D4^-/2C11^-$ cells. Separation of these two populations was carried out by FAST Systems, Inc. (Gaithersburg, MD) using electronic cell sorting after staining with fluorescein-conjugated 2C11. The 7D4 adherent population was released from plates by scraping (average yield of 9.7%).

FCM analysis. Thymocytes from day 15 to 18 embryos were stained sequentially with FITC-conjugated mAb, biotin-conjugated mAb, and Texas red streptavidin. Control groups were stained with fluorescein- or biotin-conjugated anti-human Leu 4 mAb. FCM analysis was performed as previously described (20) using a modified Becton-Dickinson Dual Laser FACS II equipped with the manufacturer's filters and photomultiplier tubes, an argon ion laser for fluorescein excitation and a pumped dye laser for Texas-Red excitation, and interfaced to a Digital Equipment Corporation PDP 11/84 computer. All analyses were performed using three-decade logarithmic amplification and data were collected and analysed using software designed by the Computer Systems Laboratory of the Division of Computer Research and Technology at the NIH. Fluorescence data were collected on viable cells as determined by forward light scatter intensity and propidium iodide exclusion. The percent of cells positive for a given mAb was calculated by subtracting the number of cells staining with a control mAb from the number of cells positive with the experimental mAb.

Assays of cell proliferation. The 10^5 cells were cultured in round-bottomed microtiter plates in 200 μl of DMEM supplemented with nonessential amino acids, 10% FCS, 1 mM sodium pyruvate, 10 mM HEPES, 5×10^{-5} M 2-ME, and 1 mM glutamine. Plates were incubated for 3 days at 37°C in humidified atmosphere containing 5% CO_2 in air, in the presence of indicated reagents. Cultures were pulsed overnight with 1 μCi [^{3}H]TdR/well (New England Nuclear, Boston, MA), harvested and counted for incorporated ^{3}H. For stimulation with anti-CD3, microtiter wells were coated by incubation for 1 h at room temperature with the mAb 145-2C11 at a concentration of 1 μg/ml in 0.01 M borate buffered saline, pH 8.5, and were then washed before addition of cells. rIL-2 was used at a final concentration of 50 U/ml unless otherwise indicated. PMA was used at a final concentration of 10 ng/ml and ionomycin at 20 ng/ml. In assays of lymphokine production, 10^5 fetal thymocytes were incubated with indicated stimuli for 24 to 96 h and supernatants were harvested and assayed for lymphokine content. Culture supernatants were assayed for IL-2 or IL-4 content by using CTLL or HT2 indicator cell lines. The 5×10^3 HT2 cells in a volume of 100 μl were added to 100 μl of serially diluted test supernatants. A total of 40 h later cultures were pulsed for 8 h with 1 μCi [^{3}H]TdR. Anti-IL-2 and anti-IL-4 mAb specifically inhibited the response of indicator cells to rIL-2 and rIL-4, respectively, without inhibiting the response to the irrelevent lymphokine. All results are expressed as the mean cpm of triplicate cultures as determined by liquid scintillation counting.

RESULTS

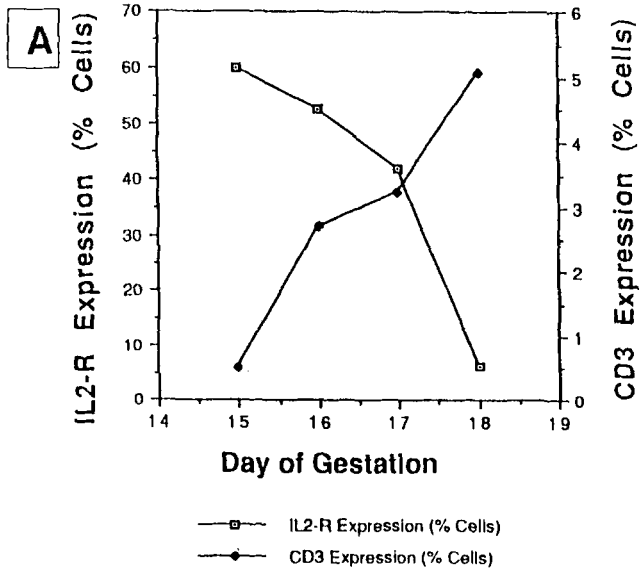
Expression of CD3 and IL-2Rα by fetal thymocytes. Fetal thymocytes were isolated from $CD2F_1$ mice and were stained with anti-CD3 or anti-IL-2Rα mAb. During fetal maturation, from day 15 to 18 of gestation, the percent of thymocytes expressing IL-2Rα decreased progressively, whereas the percent expressing CD3 increased (Fig. 1*A*), confirming previously published data (21, 22). The total number of cells contained in the fetal thymus increased 80-fold over this period, so that the absolute number of $IL\text{-}2R\alpha^+$ thymocytes increased approximately 1000-fold from day 15 to 18, and the absolute number of $CD3^+$ cells actually increased approximately 8-fold. Strikingly, two-color analysis demonstrated that IL-2Rα and CD3 are expressed on fetal thymocytes in a mutually exclusive fashion over the course of fetal maturation (Fig. 1*B*). Three distinct thymocyte subpopulations were identified: $IL\text{-}2R\alpha^+/CD3^-$ (52.6% of day-16 thymocytes), $IL\text{-}2R\alpha^-/CD3^+$ (2.7% of day 16 cells), and $IL\text{-}2R\alpha^-/CD3^-$ (44.5% of day 16 cells); no $IL\text{-}2R\alpha^+/CD3^+$ cells were detected (Fig. 1*B*).

Separation of $IL\text{-}2R\alpha^+/CD3^-$, $IL\text{-}2R\alpha^-/CD3^+$, and $IL\text{-}2R\alpha^-/CD3^-$ fetal thymocyte subpopulations. $IL2\text{-}R\alpha^+$ and $IL\text{-}2R\alpha^-$ cells were separated from day-16 fetal thymocytes by panning on tissue culture dishes that had been coated with anti-IL-2Rα mAb (7D4). The 7D4-adherent population was 94.4% $IL\text{-}2R\alpha^+$ and 0.1% $CD3^+$ when analyzed by FCM (Fig. 2), and is designated as the $IL\text{-}2R\alpha^+/CD3^-$ subpopulation. The 7D4-non-adherent population, after treatment with 7D4 and C′ to eliminate residual $7D4^+$ ($IL\text{-}2R\alpha^+$) cells, was 2.8% $CD3^+$ and 0.6% $IL\text{-}2R^+$. 7D4-non-adherent cells were further fractionated by flow cytometry into $CD3^+$ and $CD3^-$ populations, yielding one fraction that was 97.6% $IL\text{-}2R\alpha^-/CD3^-$ and 0.0% $CD3^+$ (designated the $IL\text{-}2R\alpha^-/CD3^-$ fraction), and a second fraction (designated $IL\text{-}2R\alpha^-/CD3^+$) that was presumably highly enriched in $CD3^+$ cells, but was recovered in numbers insufficient to allow flow cytometric re-analysis. Detection of IL-2Rα was carried out in these studies by indirect staining with mouse anti-rat Ig antibody to avoid blocking of IL-2R detection by 7D4 antibody used for fractionation.

Phenotypic analysis of fetal thymocyte subpopulations. The subpopulations of 16-day fetal thymocytes defined by IL-2Rα and CD3 expression were analyzed for expression of Thy-1, CD4, CD8, and CD5. Thy-1 was expressed on 95.4% of day-16 thymocytes, CD5 on 46.3%, and CD8 on 14.5%; no subpopulation of $CD4^+$ cells was detected (data not shown). Dual parameter analysis demonstrated that essentially all $CD8^+$ cells were $CD3^-$ and $IL\text{-}2R\alpha^-$ (data not shown). CD5 expression was somewhat higher on $IL\text{-}2R\alpha^-$ cells than on $IL\text{-}2R\alpha^+$ cells, and was highest on the small population of $CD3^+$ cells present in day-16 thymocytes (data not shown).

Functional responsiveness of fetal thymocyte subpopulations. Fetal thymus subpopulations were also examined for their responses to anti-CD3 and rIL-2. No population responded to either anti-CD3 alone or to IL-2 alone (Fig. 3). Unfractionated day-16 fetal thymocytes and 7D4-nonadherent ($IL\text{-}2R\alpha^-/CD3^+$ and $IL\text{-}2R\alpha^-/CD3^-$) thymocytes proliferated in response to anti-CD3 plus rIL-2; 7D4-adherent ($IL\text{-}2R\alpha^+/CD3^-$) thymocytes did not respond to anti-CD3, even in the presence of widely titrated concentrations of rIL-2 (Fig. 3*A* and data not shown). Further fractionation of 7D4-nonadherent cells by flow cytometry demonstrated that both $IL\text{-}2R\alpha^-/CD3^-$ and $IL\text{-}2R\alpha^-/CD3^+$ cells proliferated in response to anti-CD3 plus rIL-2 (Fig. 3 *B* and *C*). All subpopulations proliferated in response to PMA + ionomycin + rIL-2, indicating that differences in response to anti-CD3 + IL-2 were not due to generalized differences in proliferative capacity in vitro (Fig. 4). These responses to PMA + ionomycin were completely or partially dependent on exogenous IL-2 (data not shown).

To determine the effect of activation by anti-CD3 + IL-2 on cell surface phenotype, 7D4-adherent, 7D4-non-



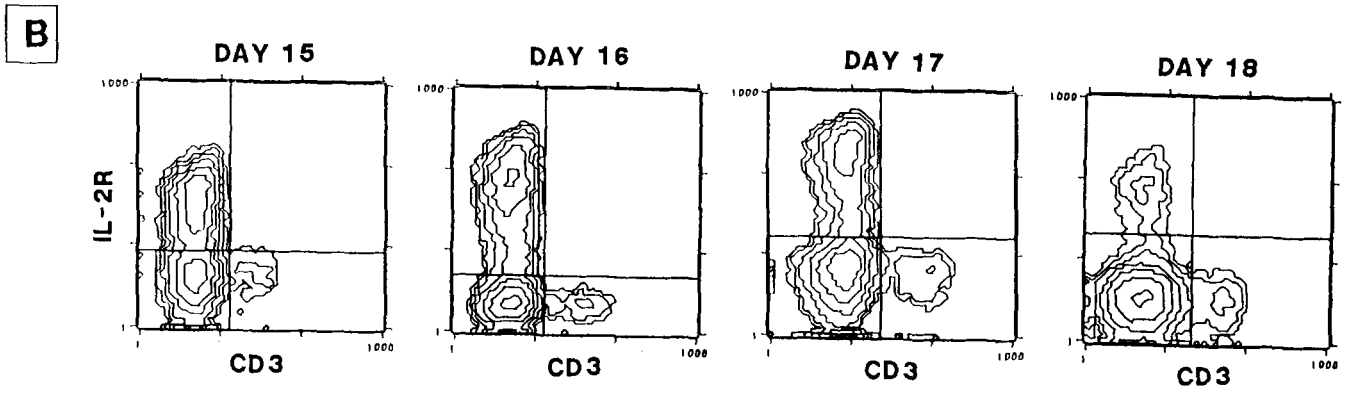


Figure 1. Expression of IL-2Rα and TCR/CD3 on fetal thymocytes. Thymocytes were prepared from day 15 to 18 fetuses and stained sequentially with fluorescein-conjugated anti-CD3ε mAb 2C11 and biotin-conjugated anti-IL-2Rα mAb 7D4 as described in *Materials and Methods*. *A*, The percentage of cells staining positively for CD3 or IL-2R is expressed as a function of gestational age of thymocytes. *B*, Dual parameter staining of fetal thymocytes with anti-CD3 and anti-IL-2Rα mAb. The indicated markers were selected based upon staining with control mAb.

adherent, and flow cytometrically isolated IL-2Rα$^-$/CD3$^-$ thymocytes were stimulated by culturing for 5 days with anti-CD3 + IL-2 and were then analyzed by flow cytometry. Survival of 7D4-adherent cells was extremely poor and did not allow cytometric analysis of cultured cells. In contrast, this stimulation induced in unfractionated cells, in 7D4-nonadherent cells and in IL-2Rα$^-$/CD3$^-$ cells the appearance of a phenotypically new population of cells that expressed both IL-2Rα and CD3 (Fig. 5). The precursors of this population could include IL-2Rα$^-$/CD3$^+$ and IL-2Rα$^-$/CD3$^-$ cells, both of which are present in the 7D4 non-adherent population. It is unlikely that the appearance of an IL-2Rα$^+$/CD3$^+$ population reflects differentiation of contaminating IL-2Rα$^+$/CD3$^-$ cells because the 7D4-adherent population that is enriched in this phenotype did not generate this phenotypic change in response to anti-CD3 + IL-2 (Fig. 5). The unresponsiveness of 7D4-adherent cells to IL-2 alone or in combination with anti-CD3 did not appear to reflect blocking of IL-2R by 7D4 (anti-IL-2Rα) antibody that might have been bound during fractionation on 7D4-coated plates, because these cells were susceptible to staining with biotin-conjugated 7D4 antibody and proliferated in response to rIL-2 in the presence of PMA and ionomycin (data not shown). In addition, 7D4-non-adherent cells but not 7D4-adherent (IL-2Rα$^+$) cells proliferated in response to IL-4 plus anti-CD3 (data not shown). Stimulation with PMA + ionomycin did not induce the appearance of IL-2Rα$^+$/CD3$^+$ cells in any population of fetal thymocytes (data not shown). Thus, the induction of a novel IL-2Rα$^+$/CD3$^+$ population was unique to stimulation with anti-CD3 + IL-2.

Lymphokine secretion by fetal thymocyte subpopulations. To further characterize the thymocyte subpopulations being studied, lymphokine production was evaluated by stimulating thymocytes with PMA and ionophore, and measuring the ability of supernatants from these cultures to support proliferation of lymphokine-responsive indicator cells. The concentrations of PMA + ionomycin used to stimulate fetal thymocytes did not directly stimulate proliferation of indicator HT2 cells (Fig. 6*B*). HT2 cells did proliferate significantly in response to supernatant from 7D4-nonadherent (IL-2Rα$^-$) thymocytes but not in response to supernatant from 7D4-adherent (IL-2Rα$^+$) thymocytes (Fig. 6*A*). Further analysis demonstrated that supernatants from flow cytometrically isolated IL-2Rα$^-$/CD3$^-$ thymocytes, but not supernatants from IL-2Rα$^-$/CD3$^+$ thymocytes supported HT2 proliferation (Fig. 6*B*). Similar results were obtained when supernatants were generated by thymocyte stimu-

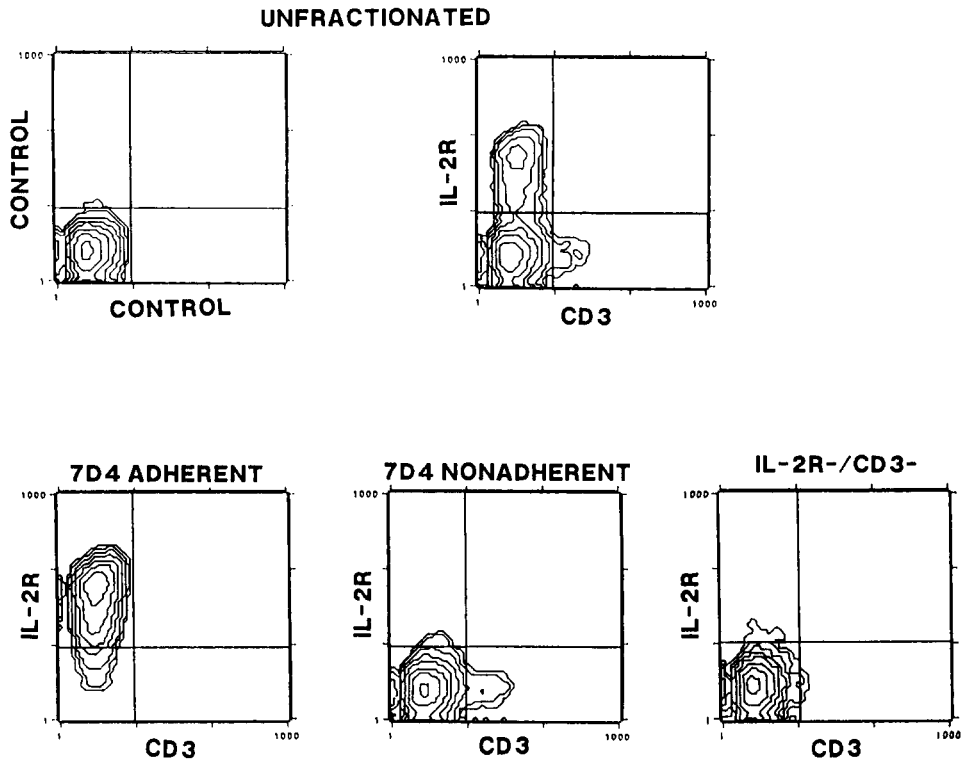


Figure 2. Expression of IL-2Rα and TCR/CD3 on fractionated day-16 fetal thymocytes. Day-16 fetal thymocytes were fractionated into 7D4 (anti-IL-2Rα mAb)-adherent and nonadherent cells by panning. 7D4-nonadherent (IL-2Rα$^-$) cells were further fractionated into IL-2Rα$^-$2C11$^-$ and IL-Rα$^-$CD3$^+$ populations by flow cytometric sorting. The number of IL-2Rα$^-$CD3$^+$ cells recovered was not sufficient to allow flow cytometric analysis. Cell populations were stained as in Figure 1 with the exception that 7D4 staining was performed by incubation of cells with 7D4 mAb, followed by washing and then incubating with biotin-conjugated goat anti-rat Ig antibody.

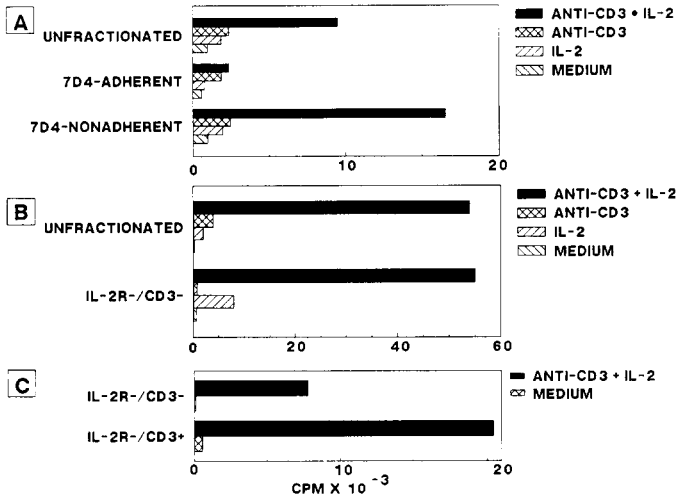


Figure 3. Proliferative responses of day-16 fetal thymocytes to anti-CD3 and IL-2. Day-16 fetal thymocytes were fractionated into 7D4 (anti-IL-2Rα mAb) adherent and nonadherent cells by panning. 7D4-nonadherent (IL-2Rα$^-$) cells were further fractionated into IL-2Rα$^-$CD3$^-$ and IL-Rα$^-$CD3$^+$ populations by flow cytometric sorting. The 10^5 unfractionated or fractionated day 16 fetal thymocytes were cultured for 3 days in the presence or absence of immobilized anti-CD3 mAb and rIL-2 as described in *Materials and Methods*. Proliferation of thymocytes was assayed as ^{3}H-thymidine incorporation.

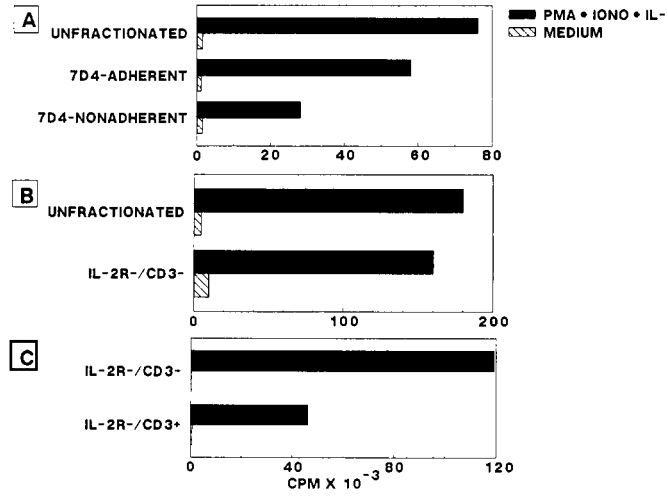


Figure 4. Proliferative responses of 7D4-adherent and 7D4-nonadherent thymocytes to PMA + Ionomycin + IL-2. Day-16 fetal thymocytes were fractionated into 7D4 (anti-IL-2Rα mAb)-adherent and nonadherent cells by panning. 7D4-nonadherent (IL-2Rα$^-$) cells were further fractionated into IL-2Rα$^-$CD3$^-$ and IL-Rα$^-$CD3$^+$ populations by flow cytometric sorting. The 10^5 cells from each fraction were cultured for 3 days in the presence or absence of PMA + Ionomycin + rIL-2. Proliferation of thymocytes was assayed as ^{3}H-thymidine incorporation.

lation in the presence of added 7D4 antibody to block consumption of IL-2 (23, 24) (data not shown). The proliferation of HT2 cells in response to thymocyte supernatants was completely blocked by the addition of anti-IL-2 mAb (S4B6) and was not affected by the addition of anti-IL-4 mAb (11B11) (Fig. 6*A*), indicating that IL-2 is the predominant lymphokine detected in fetal thymocyte supernatants in these assays.

DISCUSSION

Day-15 to 16 fetal thymocytes, which are predominantly CD4$^-$CD8$^-$, represent a population of immature cells that contains precursors of the mature T cell lineage. The small population of CD3$^-$CD4$^-$8$^+$ cells identified in the present study appears to represent the immature "transitional" population previously described (1) rather than a mature single positive population. Overall, fetal thymocytes are heterogeneous, as defined by the expression of cell surface molecules and by in situ cell cycle status (1), suggesting that they contain a number of functionally distinct subpopulations. However, the ontogenic and functional relationships among these subsets are not fully defined. In an attempt to analyze this complexity, we have studied the fetal thymocyte populations

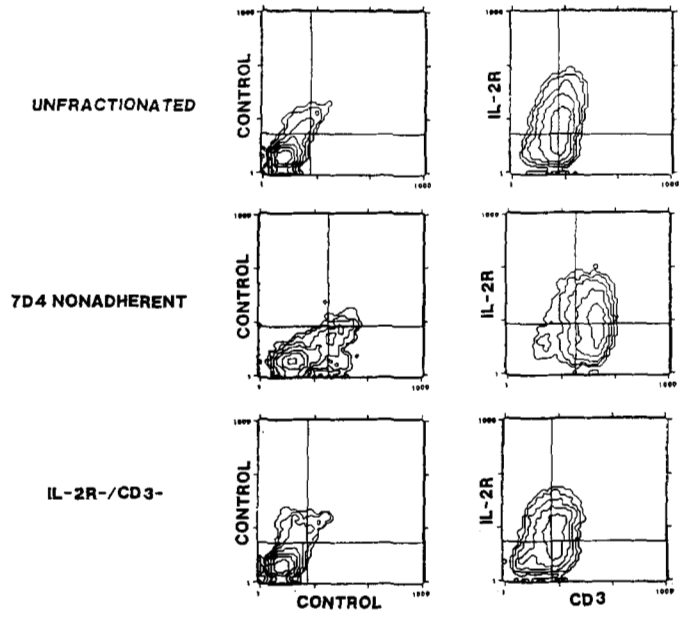


Figure 5. Phenotypic change induced in fetal thymocytes by stimulation with anti-CD3 + IL-2. Day-16 fetal thymocytes were fractionated into 7D4 (anti-IL-2Rα mAb)-adherent and nonadherent cells by panning. IL-2Rα$^-$2C11$^-$ cells were isolated from the 7D4-nonadherent population by flow cytometric sorting. Unfractionated or fractionated thymocytes were cultured for 5 days in the presence of immobilized anti-CD3 mAb and rIL-2, and cultured cells were then stained with anti-CD3 and anti-IL-2Rα or with control mAb.

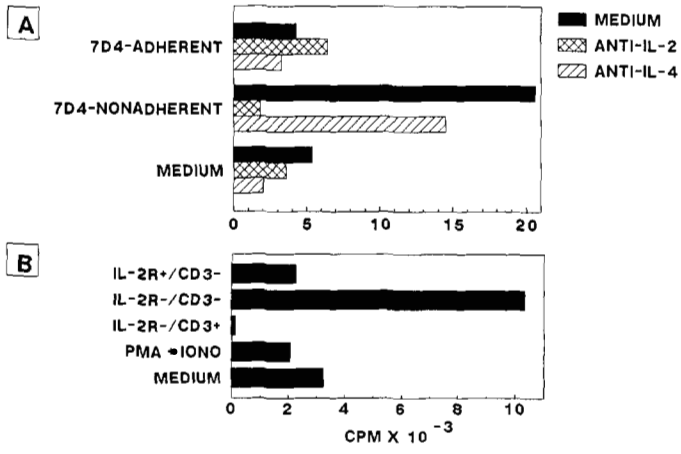


Figure 6. Lymphokine production by day-16 fetal thymocytes. Day-16 fetal thymocytes were fractionated into 7D4 (anti-IL-2Rα mAb)-adherent and nonadherent cells by panning. 7D4-nonadherent (IL-2Rα$^-$) cells were further fractionated into IL-2Rα$^-$CD3$^-$ and IL-Rα$^-$CD3$^+$ populations by flow cytometric sorting. The 10^5 unfractionated or fractionated thymocytes were cultured for 48 h with PMA + Ionomycin in a culture volume of 200 μl. At the end of this culture, supernatant was harvested and added in titrated concentrations to HT2 indicator cells in the presence or absence of anti-IL-2 or anti-IL-4 mAb. Proliferation of the indicator HT2 cells was assayed as ^{3}H-thymidine incorporation.

defined by expression of TCR (CD3) and by the α-(p55) chain of the IL-2R. This report describes the existence of three distinct subpopulations of fetal thymocytes as defined by their expression of IL-2Rα and CD3, and demonstrates that these subpopulations are also distinct from one another in terms of their activation requirements and functional capacity for lymphokine secretion.

The three subpopulations of fetal thymocytes identified in the present study were 1) IL-2Rα$^+$/CD3$^-$, 2) IL-2Rα$^-$/CD3$^+$, and 3) IL-2Rα$^-$/CD3$^-$. Strikingly, this work demonstrated expression of IL-2Rα and of CD3 were mutually exclusive; i.e., no IL-2Rα$^+$/CD3$^+$ cells were detected in freshly isolated thymocytes. This finding was consistent over the course of fetal maturation from day 15 through 18, during which time the proportion of CD3$^+$ cells progressively increased and the proportion of IL-2Rα$^+$ cells decreased. The novel finding of mutual exclusivity in expression of CD3 and IL-2Rα is potentially important to understanding the nature of signals delivered to T cells during intra-thymic development. Previous reports demonstrated that fetal thymocytes express high levels of cell surface IL-2R (22, 23), leading to speculation that such expression might be induced during thymic development as a result of reaction of immature thymocytes with thymic self Ag. However, substantial IL-2Rα expression actually precedes the appearance of cell surface TCR/CD3 expression. Moreover, the finding presented here that, even after cell surface TCR/CD3 expression appears, IL-2Rα is not expressed on CD3$^+$ cells suggests that the stimulus to IL-2Rα expression during intra-thymic T cell development may not involve stimulation through the TCR. In vitro stimulation of fetal thymocytes with anti-CD3 mAb and IL-2 resulted in the appearance of a substantial proportion of IL-2Rα$^+$/CD3$^+$ cells, indicating that under appropriate conditions such a population can be induced. The failure to identify this population in freshly isolated fetal thymocytes at any stage of development suggests that equivalent stimulation through TCR/CD3 and IL-2R does not occur in vivo, or that other events prevent the induction and/or accumulation of these cells.

The anti-CD3 mAb used here to detect TCR expression is reactive with TCR complexes consisting of either αβ or γδ heterodimers. In the current studies, it was demonstrated by immunoprecipitation of day-15 to 16 fetal thymocytes that, at this stage of development, CD3 is associated with γ and δ TCR chains, but with no detectable TCR α or β (data not shown), consistent with previous reports (1). IL-2R expression in the present study was analyzed by reactivity with an antibody (7D4) that is specific for the α or p55 chain of the mouse IL-2R. At least three forms of the IL-2R are known to exist: a high affinity receptor consisting of α (p55) and β (p70–75) chains, an intermediate affinity receptor consisting of β-chain in the absence of α, and a low affinity form consisting of α-chain without β (24). Only the high and intermediate affinity receptors, both of which involve β-chain expression, appear to be capable of internalization and signal transduction in response to IL-2. To date, no structural characterization of the IL-2R expressed on immature thymocytes has been reported, and it is not possible at present to assess the functional status of IL-2R expression on murine thymocytes based on serologic means. Previous studies have reported that immature thymocytes do not internalize IL-2 or proliferate in vitro in response to IL-2 stimulation (6), raising questions about the functional role of IL-2 and IL-2R in immature thymocytes. In addition, apparently normal human T cell development has been reported to occur in a patient without detectable IL-2 production (25). In contrast, it has been reported that anti-IL-2Rα mAb strongly inhibits intra-thymic differentiation both in vivo and in thymic organ culture (11, 12), a finding that supports a physiologic role for endogenous IL-2 in thymic differentiation; in similar studies, others have failed to find inhibition by

anti-IL-2Rα (13). In the present study, IL-2Rα$^+$ fetal thymocytes did not proliferate in response to IL-2 alone. However, the response to PMA plus ionophore was significantly augmented by the addition of rIL-2 (Fig. 6), consistent with previous reports (6). It cannot be distinguished from these results whether the IL-2R expressed on thymocytes in vivo is capable of transducing a signal that acts synergistically with those delivered by PMA and ionophore, or whether stimulation with PMA and ionophore induces the expression of a functional IL-2R.

The identification and isolation of subpopulations of fetal thymocytes based on their expression of IL-2Rα and CD3 allowed a comparison of their functional capacities. This comparison resulted in several novel findings. In response to anti-CD3 plus rIL-2, IL-2Rα$^-$/CD3$^+$ and IL-2Rα$^-$/CD3$^-$ cells proliferated strongly, whereas IL-2Rα$^+$/CD3$^-$ cells did not respond. In addition, stimulation of IL-2Rα$^-$ populations with anti-CD3 and rIL-2 resulted in the appearance of a substantial proportion of cells that were IL-2Rα$^+$/CD3$^+$, a phenotype that was never detected in freshly isolated fetal thymocytes. The IL-2Rα$^-$ populations used here are probably quite heterogeneous, and the identity of the subset of cells responding to anti-CD3 + rIL-2 is not clear. In this respect, the ability of IL-2Rα$^-$/CD3$^-$ cells to respond to anti-CD3 plus IL-2 is particularly intriguing. A proportion of cells in this population might express levels of CD3 that were undetectable by flow cytometry or immunoprecipitation (26). Activation of these cells by anti-CD3 might then up-regulate their CD3 expression, as well as induce expression of a functional IL-2R. Alternatively, some IL-2Rα$^-$/CD3$^-$ cells might express sufficient functional IL-2R (e.g., intermediate affinity β-chains that were not detected in our analysis with α chain-specific mAb) to mediate activation by IL-2, resulting in the induction of TCR/CD3 expression which then provided a target for anti-CD3 signaling. A third possibility is that some IL-2Rα$^-$/CD3$^-$ fetal thymocytes are pre-programed to express either IL-2R or CD3/TCR, and that this expression occurred during in vitro culture, with consequent responsiveness to anti-CD3 + rIL-2. The subsets of fetal thymocytes characterized here also differed in their capacity to secrete lymphokines. Only IL-2Rα$^-$/CD3$^-$ cells, and not IL-2Rα$^-$/CD3$^+$ or IL-2Rα$^+$/CD3$^-$ cells, secreted IL-2 in response to phorbol ester and ionomycin.

Recently proposed models of maturation of CD3$^-$/CD4$^-$/CD8$^-$ thymocytes suggest that cells of this phenotype progress from a stage at which they are IL-2Rα$^-$, through a transient IL-2Rα$^+$ stage, to another IL-2Rα$^-$ state before further differentiation (27). Moreover, in vitro studies as well as in vivo adoptive transfer experiments have identified both IL-2R$^+$ and IL-2R$^-$ populations of CD3$^-$/CD4$^-$/CD8$^-$ cells as precursors of the T cell lineage (28–31). Cells with progenitor activity most probably represent only small subpopulations of the IL-2R$^+$ and IL-2R$^-$ populations that were studied in the present work, so that the functional characterization presented here may not directly reflect the properties of these precursors. Nevertheless the data presented here suggest that IL-2R$^+$ and IL-2R$^-$ precursor populations may differ substantially in their activation requirements and functional capacities.

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REFERENCES

1. **Fowlkes, B. J., and D. M. Pardoll.** 1989. Molecular and cellular events of T cell development. *Advances in Immunol. 44:207.*
2. **Raulet, D. H.** 1985. Expression and function of interleukin-2 receptors on immature thymocytes. *Nature 314:101.*
3. **von Boehmer, H., A. Crisanti, P. Kisielow, and W. Haas.** 1985. Absence of growth by most receptor-expressing fetal thymocytes in the presence of interleukin-2. *Nature 314:539.*
4. **Hardt, C., S. Fleischer, M. Steinmetz, and H. Wagner.** 1986. Detection of rearranged T cell receptor β-chain gene and induction of cytolytic function in interleukin 2-responsive day 14–15 murine fetal thymocytes. *Eur. J. Immunol. 16:1087.*
5. **Palacios, R., and H. von Boehmer.** 1986. Requirements for growth of immature thymocytes from fetal and adult mice in vitro. *Eur. J. Immunol. 16:12.*
6. **Lowenthal, J. W., R. C. Howe, R. Ceredig, and H. R. MacDonald.** 1986. Functional status of interleukin 2 receptors expressed by immature (Lyt-2$^-$/L3T4$^-$) thymocytes. *J. Immunol. 137:2579.*
7. **Ceredig, R.** 1986. Proliferation in vitro and interleukin production by day 14 fetal and adult Lyt-2$^-$/L3T4$^-$ mouse thymocytes. *J. Immunol. 137:2260.*
8. **Skinner, M., G. Le Gros, J. Marbrook, and J. D. Watson.** 1987. Development of fetal thymocytes in organ culture. Effect of interleukin 2. *J. Exp. Med. 165:1481.*
9. **Takei, F.** 1988. IL-2 and IL-4 stimulate different subpopulations of double-negative thymocytes. *J. Immunol. 141:1114.*
10. **Suda, T., R. Murray, C. Guidos, and A. Zlotnik.** 1990. Growth-promoting activity of IL-1α, IL-6, and tumor necrosis factor-α in combination with IL-2, IL-4, or IL-7 on murine thymocytes. Differential effects on CD4/CD8 subsets and on CD3$^+$/CD3$^-$ double-negative thymocytes. *J. Immunol. 144:3039.*
11. **Jenkinson, E. J., P. Jhittay, R. Kingston, and J. J. T. Owen.** 1987. Importance of IL-2 receptors in intra-thymic generation of cells expressing T-cell receptors. *Nature 329:160.*
12. **Tentori, L., D. L. Longo, J. C. Zuniga-Pflucker, C. Wing, and A. M. Kruisbeek.** 1988. Essential role of the interleukin 2-interleukin 2 receptor pathway in thymocyte maturation in vivo. *J. Exp. Med. 168:1741.*
13. **Plum, J., and M. De Smedt.** 1988. Differentiation of thymocytes in fetal organ culture: lack of evidence for the functional role of the interleukin 2 receptor expressed by prothymocytes. *Eur. J. Immunol. 18:795.*
14. **Malek, T. R., R. J. Robb, and E. M. Shevach.** 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA 80:5694.*
15. **Leo, O. M., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone.** 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA 84:1374.*
16. **Pierres, A., P. Nuquet, A. van Aghtoven, F. Bekkhoucha, F. Denizot, Z. Mishal, A. M. Schmitt-Verhulst, and M. Pierres.** 1984. A rat anti-mouse T4 monoclonal antibody (H129.19) inhibits the proliferation of Ia reactive T cell clones and delineates two phenotypically distinct (T4$^+$,Lyt-2,3$^-$ and T4$^-$,Lyt-2,3$^+$) subsets among anti-Ia cytolytic T cell clones. *J. Immunol. 132:2775.*
17. **Ohara, J., and W. E. Paul.** 1985. Production of a monoclonal antibody to and characterization of B-cell stimulatory factor-1. *Nature 315:333.*
18. **Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Geidlin, and R. A. Coffman.** 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine production and secreted proteins. *J. Immunol. 136:2348.*
19. **Unkeless, J. C.** 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med. 150:580.*
20. **Segal, D. M., S. O. Sharrow, J. F. Jones, and R. P. Siraganian.** 1981. Fc (IgG) receptors on rat basophilic leukemia cells. *J. Immunol. 126:138.*
21. **Habu, S., K. Okumura, T. Diamantstein, and E. M. Shevach.** 1985. Expression of interleukin 2 receptor on murine fetal thymocytes. *Eur. J. Immunol. 15:456.*
22. **Ceredig, R., J. Lowenthal, M. Nabholz, and R. MacDonald.** 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature 314:98.*
23. **Mizuochi, T., S. Ono, T. R. Malek, and A. Singer.** 1986. Characterization of two distinct primary T cell populations that secrete interleukin 2 upon recognition of class I or class II major histocompatibility antigens. *J. Exp. Med. 163:603.*
24. **Smith, K. A.** 1987. The structure of high affinity IL-2 receptors: α and β chains. *Immunol. Today 8:11.*

25. **Disanto, J. P., C. A. Keever, T. N. Small, G. L. Nichols, and R. J. O'Reilly.** 1990. Absence of interleukin 2 production in a severe combined immunodeficiency disease syndrome with T cells. *J. Exp. Med. 171:1697.*
26. **Nikolic-Zugic, J., and M. W. Moore.** 1989. T cell receptor expression on immature thymocytes with in vivo and in vitro precursor potential. *Eur. J. Immunol. 19:1957.*
27. **Pearse, M., L. Wu, M. Egerton, A. Wilson, K. Shortman, and R. Scollay.** 1988. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA 86:1614.*
28. **Hyman, R., J. Lesley, R. Schulte, and J. Trotter.** 1986. Progenitor cells in the thymus: most thymus-homing progenitor cells in the adult mouse thymus bear Pgp-1 glycoprotein but not interleukin-2 receptor on their cell surface. *Cell. Immunol. 101:320.*
29. **Crispe, I. N., M. W. Moore, L. A. Husmaann, L. Smith, M. J. Bevan, and R. P. Shimonkevitz.** 1987. Differentiation potential of subsets of $CD4^-8^-$ thymocytes. *Nature 329:336.*
30. **Nakano, N., R. R. Hardy, and T. Kishimoto.** 1987. Identification of intrathymic T progenitor cells by expression of Thy-1, IL 2 receptor and CD3. *Eur. J. Immunol. 17:1567.*
31. **Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pearse, L. Wu, and K. Shortman.** 1988. Developmental status and reconstitution potential of subpopulations of murine thymocytes. *Immunol. Rev. 104:81.*