ESSENTIAL ROLE OF THE INTERLEUKIN 2-INTERLEUKIN 2 RECEPTOR PATHWAY IN THYMOCYTE MATURATION IN VIVO

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Antigen activation of mature T cells results in synthesis and secretion of IL-2 and surface expression of functional IL-2 receptors (IL-2-R). Subsequent binding and endocytosis of IL-2 leads to T cell growth (1, 2). IL-2-R have also been found on a high proportion of the small subset of $CD4^-$,$CD8^-$ adult thymocytes and on day 14–16 fetal $CD4^-$,$CD8^-$ thymocytes in mice (3–8). These $CD4^-$,$CD8^-$ thymocytes have the properties of intrathymic "stem cells," in that they are the first to appear in the embryonic thymus (9), and contain the precursors to all major T cell subsets (10). The presence of IL-2-R on intrathymic precursors has led to the speculation that the IL-2–IL-2-R pathway might be involved in the proliferation and/or differentiation of T cells. However, the physiological relevance of IL-2-R expression by $CD4^-$,$CD8^-$ thymocytes is unclear, as the receptors appear to be nonfunctional: freshly isolated $CD4^-$,$CD8^-$ adult or fetal thymocytes do not respond to IL-2 (4, 6–8), and express only low affinity IL-2-R (3, 8). To test the hypothesis that the IL-2–IL-2-R pathway plays a role in intrathymic fetal T cell development, we attempted to block signaling via IL-2-R occupancy in the fetal thymus by treating pregnant mice with mAbs to the IL-2-R. The results demonstrate that thymocytes from newborn mice born to anti-IL-2-R antibody-treated mothers do not contain mature $CD4^+$ or $CD8^+$ single-positive T cells, which suggests that IL-2–IL-2-R interactions provide signals crucial to in vivo intrathymic development of T cells.

Materials and Methods

Mice and mAb Treatments. Timed pregnant C57 BL/6 mice were treated daily from day 12 of pregnancy with 2 mg each of the monoclonal rat anti-mouse IL-2-R mAbs 3C7 and 2E4 or 3C7 and 7D4 (11). Purified antibodies were prepared from ascites, and antibody injections were given intraperitoneally. Control mice received daily injections of purified rat IgG or saline, once it was established that purified rat IgG had no effect.

Flow Cytometry Analysis. Thymocyte suspensions from fetal or newborn mice were prepared in HBSS without phenol red containing 0.1% BSA and 0.1% sodium azide. Staining and washing procedures were as described (10). Flow cytometry analysis was performed on a dual laser FACS II (B-D Automated Immunochemistry, Salt Lake City, UT) interfaced

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to a PDP 11/24 computer (Digital Equipment Corp., Maynard, MA). Data were collected on 50,000 viable cells, as determined by forward light scatter and propidium iodide gating. For IL-2-R analysis, cells were reacted either with a combination of FITC-labeled mouse mAb to rat-κ chain (B-D Automated Immunochemistry) and FITC-labeled goat anti-rat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), or with 3C7 plus 2E4, followed by the FITC-anti-rat IgG reagents. For CD8/CD4 and CD5/CD8 analysis (10), cells were reacted with FITC-conjugated anti-CD8 mAb (B-D Automated Immunochemistry) followed by biotin-conjugated anti-CD4 mAb, followed by Texas Red streptavidin (TRA) (Bethesda Research Laboratories, Bethesda, MD), or by FITC-conjugated anti-CD5 mAb, followed by biotin-conjugated anti-CD8 mAb and TRA. For Vβ8 analysis, cells were reacted with the Vβ8 chain-specific mAb F23.1 (12), followed by FITC-anti-mouse IgG2a, or with FITC-anti-mouse IgG2a alone.

Limiting Dilution Microculture Techniques. Thymocytes from newborn mice were tested for frequency of IL-2-producing cells and CTL precursor cells (9, 13). For IL-2 precursor assays, supernatants of microtiter wells were tested after 6 d for their ability to support proliferation of CTLL cells. For CTL precursor assays, 10^3 ^{51}Cr-labeled P815 (*H-2*^{d}) target cells were added per well after 7 d; radioactivity in the supernatants was measured after 6 h at 37°C.

Results and Discussion

Fetal thymocytes from anti-IL-2-R mAb-treated pregnant mice bore the injected rat mAb on their surface. Fig. 1 shows that fetal day 17 thymocytes from treated mice exhibited only slightly more fluorescence when stained with 3C7 + 7D4 mAb followed by FITC-conjugated anti-rat Ig, than when stained with FITC-conjugated anti-rat Ig alone. All the rat mAb was contained within the subpopulation of dull-CD5-expressing cells (data not shown), consistent with the notion that the mAb bound to $CD4^-$,$CD8^-$ cells. Similar results were obtained at earlier time points of embryonic development, i.e., from day 13 on; even at day 20 (the time of birth), the small number of IL-2-R-bearing cells was unchanged, albeit all IL-2-R was still blocked with rat mAb (data not shown). These findings not only demonstrate that the IL-2-R can be saturated with mAb in the fetal thymus, but, even more impor-

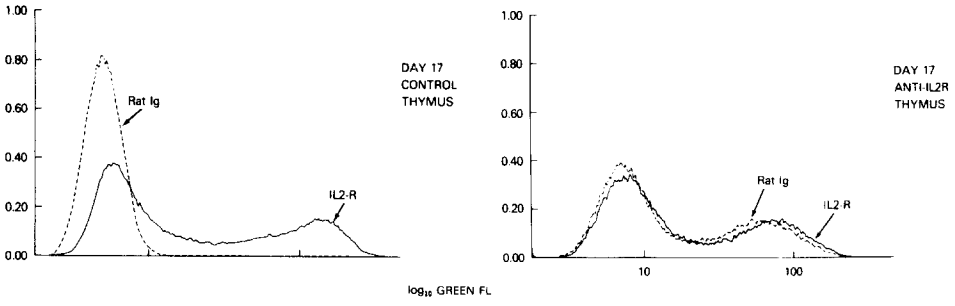

FIGURE 1. Single-color fluorescence flow cytometry (FCM) analysis of IL-2-R expression on day 17 fetal thymocytes from control (*left*) and anti-IL-2-R-treated (*right*) pregnant mothers. Timed pregnant C57Bl/6 mice were treated with a combination of rat anti-IL-2-R mAbs (3C7 plus 2E4 [11, 12]) purified from ascites. Intraperitoneal injections of 2 mg of each antibody were given daily from day 12 of pregnancy until the day of the experiment. Data are representative of five separate experiments. The percent of IL-2-R$^+$ cells was 37.3 ± 4.1% in the control group, and the percent of rat IgG$^+$ cells was 36.5 ± 3.9% in the treated group. Each histogram shows staining for FITC-anti-rat IgG (---) or staining with 3C7 plus 2E4, followed by FITC-anti-rat IgG (—).

tantly, that the IL-2-R-bearing cells were not eliminated by the antibody treatment. This is specifically relevant (see below) in view of the recent finding (14) that IL-2-R$^+$ thymocytes contain precursors for mature CD4 and CD8 single-positive T cells.

When thymocytes from newborns were examined for CD4, CD8, and CD5 (Ly-1) expression, significant differences were noted between those from control and anti-IL-2-R-treated mothers. Fig. 2, *left*, shows that the numbers of CD4 and CD8 single-positive cells are reduced in the thymus from mice born to treated mothers. This absence of T cells with a mature phenotype was confirmed by demonstrating absence of the bright CD5$^+$ (Ly-1^{hi}) CD8$^-$ subset (Fig. 2, *right*), corresponding mostly to CD4$^+$,CD8$^-$ cells (10), and absence of the bright CD5$^+$,CD4$^-$ subset (data not shown), corresponding to CD4$^-$,CD8$^+$ cells (10). In addition, the number of CD4$^-$,CD8$^-$ cells in the thymus from treated mice has increased (Fig. 2, *left*), but no change in the percentage of CD4$^+$,CD8$^+$ double-positive cells occurred. Post-

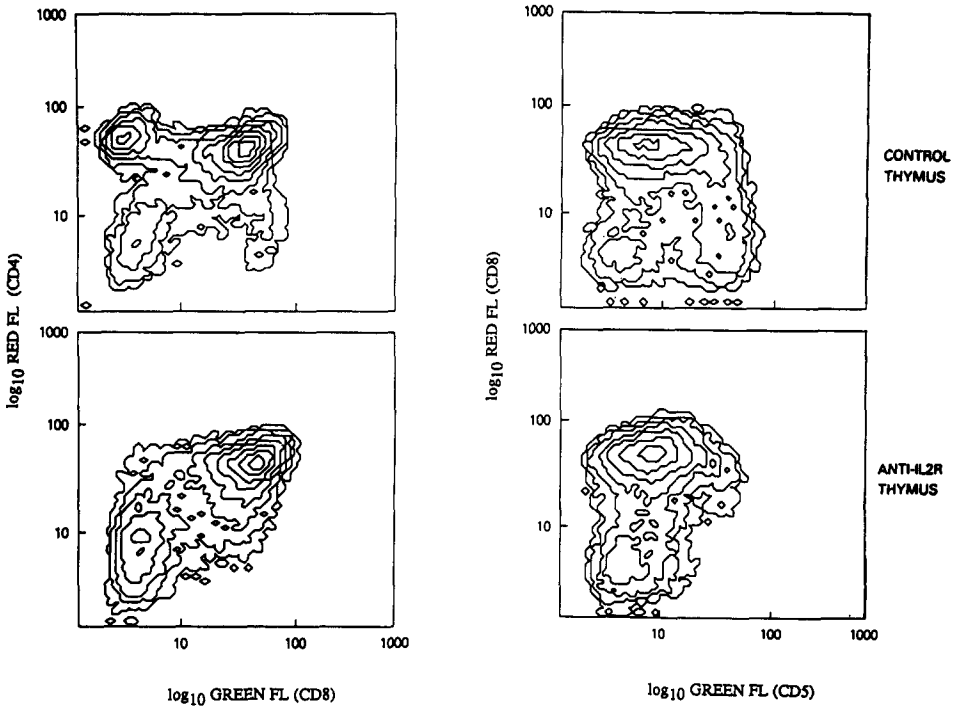

FIGURE 2. Two-color FCM analysis of cell surface CD4 and CD8 expression (*left*) or CD5 and CD8 expression (*right*) on thymocytes of newborn babies born to control (*top*) and anti-IL-R-treated (*bottom*) mothers. Pregnant mice were treated daily as indicated in Fig. 1, until the day of birth. Data are displayed as contour diagrams, with a three-decade log scale of increasing green fluorescence on the x-axis, and increasing red fluorescence on the y-axis. The x and y coordinates that defined negative cells were selected as the intersection of positive and control negative profiles in each parameter (i.e., obtained by staining with an irrelevant negative control mAb). Data are representative for five separate experiments. In this representative experiment, the percent of CD4$^+$ CD8$^-$ cells was 11.8% in control thymus, and 1.6 ± 0.4% in treated thymus; the percent of CD4$^-$,CD8$^+$ cells was 1.8% in control thymus, and 0.3% in treated thymus; the percent of bright CD5$^+$,CD8$^-$ was 10.5% in control thymus, and 1.4% in treated thymus.

natal treatment with saturating doses of anti-IL-2-R antibody had no effect on CD4/CD8 expression (data not shown; five separate experiments in which effects were monitored for up to 3 wk of life). Finally, the total number of thymocytes recovered from control and antibody-treated mothers was not different; control mice contained $10.5 \pm 2.6 \times 10^6$ cells, and treated mice, $10.8 \pm 3.2 \times 10^6$ (average of five experiments), thus excluding general toxic effects of the antibody.

These results raised the possibility that blocking of the IL-2-R in the fetal thymus prevented generation of mature T cells. To further address this question, thymocytes from control and experimental mice were tested for the presence of functional $CD4^+,CD8^-$ and $CD4^-,CD8^+$ T cells by a sensitive limiting dilution culture system, in which their ability to secrete IL-2 and generate CTL precursors in response to MHC alloantigens was determined. Functional T cells begin to appear around birth (9), reflecting appearance of a TCR-α/β heterodimer complex on single-positive cells (15). It can be seen in Table I that thymocytes from babies born to anti-IL-2-R–treated mothers are devoid of functional $CD4^+$ and $CD8^+$ T cells, in that their levels of IL-2-producing precursors and CTL precursors are below detection limits. That this lack of responsiveness was not simply due to carry-over of blocking anti-IL-2-R antibody into the cultures was deduced from the fact that control thymocytes preincubated in anti-IL-2-R antibody in vitro did not exhibit reduced precursor frequencies (data not shown). The spleens from both control and treated newborns did not contain functional T cells (data not shown), excluding the possibility that the treatment had resulted in accelerated migration of mature cells to the spleen. Thus, by both phenotypical and functional criteria, anti-IL-2-R treatment of pregnant mice results in profound depletion of mature functional $CD4^+,CD8^-$ and $CD4^-,CD8^+$ T cells in the fetal thymus.

TABLE I

Absence of Alloreactive IL-2-producing Cells and CTL Precursors in the Thymus from Anti-IL-2-R–treated Mice

Exp.	C57B1/6 responder thymus	Frequency	
		IL-2 producers	CTL precursors
1	Control	1/840	1/4210
	Anti-IL-2-R*	$<1/10^5$	$<1/10^5$
2	Control	1/670	1/3530
	Anti-IL-2-R	$<1/10^5$	$<1/10^5$
3	Control	1/720	1/3960
	Anti-IL-2-R	$<1/10^5$	$<1/10^5$

* Varying numbers of responder cells were cultured in 96-well round-bottomed microtiter plates with 5×10^4 $(B10 \times B10.D2)F_1$ nude peritoneal stimulator cells for 5 d (IL-2 precursor assay), or with 5×10^5 $(B10 \times B10.D2)F_1$ nude spleen cells and 10% delectinated rat Con A supernatant for 7 d (CTL precursor assay). Per responder cell doses, 36 replicate microcultures were used, and only experiments in which a linear semilogarithmic relationship between the proportion of negative cultures and the dose of responder cells was observed are shown. Cultures were defined as positive when the radioactivity ($[^3H]$TdR incorporation into CTLL cells for IL-2 precursor assays; ^{51}Cr release for CTL precursor assays) exceeded by >3 SD the mean radioactivity of control cultures incubated with stimulator cells alone.

Our results suggest that T cells capable of responding to stimulation through the TCR-α/β are not generated in the fetal thymus in the presence of anti-IL-2-R antibody. To more directly address this issue, we analyzed thymocytes of newborn mice from treated and untreated mothers for surface expression of TCR β chain. Since no pan-anti-murine TCR β and α chain reagents are available that detect surface protein, we used one of the TCR β chain allotype reagents. The TCR-Vβ8-specific mAb F23.1 (12) recognizes an epitope on the TCR of 20–25% of both $CD4^+$ and $CD8^+$ peripheral T cells (12, 15) in mouse strains expressing the Vβ8 gene segment. The number of TCR-Vβ8-expressing cells is fairly low in the thymus at birth (15), and F23.1 staining appears as a dull shift in staining intensity on fluorescence histograms (data not shown). Nevertheless, thymocytes from newborn control mice exhibit detectable numbers of Vβ8-expressing cells when F23.1 staining is plotted vs. light scatter, while no such cells are present in babies from the anti-IL-2-R group (Fig. 3). All Vβ8-expressing cells on the day of birth are present in the subsets expressing CD4 and/or CD8 and not among $CD4^-$,$CD8^-$ cells, in agreement with recent findings (16). However, since the total number of Vβ8-bearing cells in each subset is on the limits of reliable detection (i.e., between 0.2 and 1.0%), and only low levels of F23.1 expression were found, no percentages of Vβ8-cells for each subset can be given. These data are consistent with the absence of Vβ8-bearing cells among thymocytes from anti-IL-2-R–treated babies. Moreover, since no alloantigen-specific T cells were found in the thymus of these mice, these data, by inference, imply absence of all TCR-α/β-bearing mature T cells in the thymus from treated mice.

There is controversy over the physiological role of the IL-2-R in fetal T cell development. Previous studies have demonstrated that freshly isolated $CD4^-$,$CD8^-$, IL-$2\text{-}R^+$ thymocytes proliferate only poorly in response to IL-2 (3, 4, 6–8), are in-

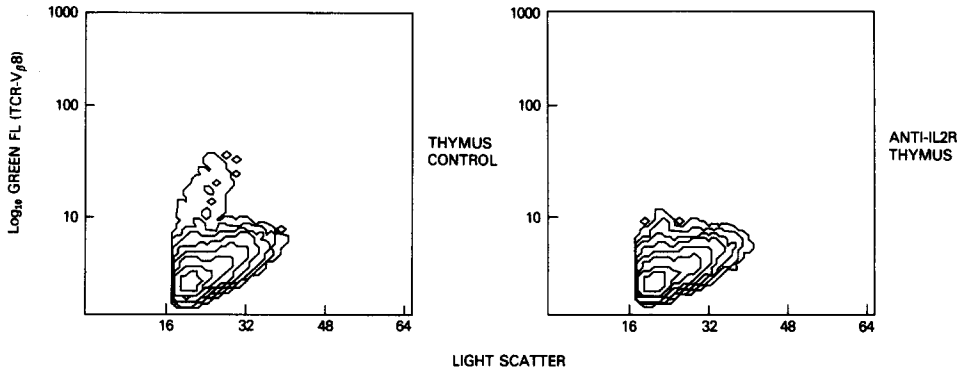

FIGURE 3. FCM analysis of TCR-Vβ8 expression by thymocytes from newborns of untreated and in vivo anti-IL-2-R mAb–treated C57BL/6 pregnant mice. Pregnant mice were treated daily from day 12 of gestation with anti-IL-2-R mAbs. Data are displayed as a contour plot, in which the log of fluorescence intensity (*y*-axis) is plotted against the light scatter (*x*-axis). No staining was obtained when control thymocytes were stained with FITC-labeled anti-mouse IgG2a alone (data not shown). The presence of rat IgG (i.e., anti-IL-2-R antibody) does not interfere with Vβ8 staining (data not shown). Two-color flow cytometry analysis of green Vβ8 staining in control mice vs. red CD4 or red CD8 staining revealed that, on the day of birth, most $V\beta8^+$ cells were in the $CD8^+$ compartment, few in the $CD4^+$ compartment, and none in the $CD4^-$,$CD8^-$ compartment.

capable of internalizing IL-2 (8), and express only low affinity IL-2-R (3, 8). Yet, it is clear that $CD4^-$,$CD8^-$ fetal thymocytes have the ability to produce IL-2 (7, 17). In addition, it is possible to induce high affinity IL-2-R on $CD4^-$,$CD8^-$ thymocytes upon activation in vitro (8). The dramatic inhibitory effects of anti-IL-2-R antibody on cell growth in thymic organ cultures (18) also suggest that, within an intact thymic microenvironment, functional IL-2-R are important. The finding that overall cell recovery of thymocytes was not affected by anti-IL-2-R treatment in vivo is not necessarily at variance with the organ culture data in which anti-IL-2-R inhibited cell growth (18); continued input of stem cells into the thymus and their subsequent expansion in vivo could overshadow inhibitory effects of anti-IL-2-R.

Since IL-2-R expression is confined to the $CD4^-$,$CD8^-$ subset (3–8), the observed consequences of anti-IL-2-R antibody treatment in our study are most likely due to effects on the $CD4^-$,$CD8^-$ subset only. These effects do not reflect elimination or inhibition of generation of the majority of IL-2-R^+ cells, and occur despite the low affinity of the IL-2-R on these cells. While our data do not formally exclude an antiproliferative or cytotoxic effect of the treatment on a small subset of IL-2-R-bearing cells, the block in maturation after anti-IL-2-R treatment is unequivocal.

Finally, although we favor an interpretation that places the important site of IL-2-R expression and action on the thymocytes themselves, it must also be acknowledged that some thymic nonlymphoid cells such as dendritic cells or macrophages could express IL-2-R. Therefore, it is possible that the maturation arrest we observed in thymocytes could result from the capacity of anti-IL-2-R antibody to block the production of an unidentified thymocyte differentiation factor normally produced by an IL-2-R-bearing accessory cell.

Summary

The role of the IL-2–IL-2-R pathway in thymocyte differentiation in vivo is unknown. We have examined fetal thymocyte development in vivo, under conditions where all IL-2-R were saturated from day 13 of gestation with anti-IL-2-R mAbs that were previously shown to render mature T cells unable to respond to IL-2. This produced a dramatic change in the composition of developing T cells: thymocytes from day 1 neonatal mice born to anti-IL-2-R-treated mothers did not contain $CD4^+$ or $CD8^+$ single-positive cell populations. In addition, no generation of surface TCR β chain–expressing T cells or antigen-reactive functional T cells occurred in treated mice. These data suggest that IL-2–IL-2-R interactions provide signals crucial to in vivo intrathymic development of mature T cells.

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References

1. Smith, K. A. 1984. Interleukin 2. *Annu. Rev. Immunol.* 2:319.
2. Weissman, A. M., J. B. Harford, P. B. Svetlik, W. J. Leonard, J. M. Depper, T. A. Wald-

mann, W. C. Greene, and R. D. Klausner. 1986. Only high-affinity receptors for interleukin 2 mediate internalization of ligand. *Proc. Natl. Acad. Sci. USA.* 83:1463.

3. Ceredig, R., J. W. Lowenthal, M. Nabholz, and H. R. MacDonald. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (Lond.).* 314:98.
4. Raulet, D. H. 1985. Expression and function of interleukin-2 receptors on immature thymocytes. *Nature (Lond.).* 314:101.
5. 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.
6. von Boehmer, H., A. Crisanti, P. Kiesilow, and W. Haas. 1985. Absence of growth by most receptor-expressing fetal thymocytes in the presence of interleukin-2. *Nature (Lond.).* 314:539.
7. Ceredig, R. 1986. Proliferation in vitro and interleukin production by 14 day fetal and adult Lyt2$^-$L3T4$^-$ mouse thymocytes. *J. Immunol.* 137:2260.
8. Lowenthal, J. W., R. C. Howe, R. Ceredig, and H. R. MacDonald. 1986. Functional status of interleukin 2 receptors expressed by immature (Lyt2$^-$ L3T4$^-$) thymocytes. *J. Immunol.* 137:2579.
9. Ceredig, R., D. P. Dialynas, F. W. Fitch, and H. R. MacDonald. 1983. Precursors of T cell growth factor producing cells in the thymus: ontogeny, frequency and quantitative recovery in a subpopulation of phenotypically mature thymocytes defined by monoclonal antibody GK1.5. *J. Exp. Med.* 158:1654.
10. Fowlkes, B. J., L. Edison, B. J. Mathieson, and T. M. Chused. 1985. Early T lymphocytes: differentiation in vivo adult intrathymic precursor cells. *J. Exp. Med.* 162:802.
11. Ortega, G. R., R. J. Robb, E. M. Shevach, and T. R. Malek. 1984. The murine IL2 receptor. I. Monoclonal antibodies that define distinct functional epitopes on activated T cells and react with activated B cells. *J. Immunol.* 133:1970.
12. Staerz, U. D., H.-G. Rammensee, J. D. Benedetto, and M. J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. *J. Immunol.* 134:3994.
13. Kruisbeek, A. M., J. J. Mond, B. J. Fowlkes, J. A. Carmen, S. Bridges, and D. L. Longo. 1985. Absence of Lyt2$^-$ L3T4$^+$ lineage of T cells in mice treated neonatally with anti-I-A correlates with absence of intrathymic I-A-bearing antigen-presenting cell function. *J. Exp. Med.* 161:1029.
14. Shimonkevitz, R. P., L. A. Husmann, M. J. Bevan, and I. N. Crispe. 1987. Transient expression of IL-2 receptor precedes the differentiation of immature thymocytes. *Nature (Lond.).* 329:157.
15. Roehm, N., L. Heron, J. Cambrier, D. DiGiusto, K. Haskins, J. Kappler, and P. Marrack. 1984. The major histocompatibility complex-restricted antigen receptor on T cells: distribution on thymus and peripheral T cells. *Cell.* 38:577.
16. Fowlkes, B. J., A. M. Kruisbeek, H. Ton-That, M. A. Weston, J. E. Coligan, R. H. Schwartz, and D. M. Pardoll. 1987. A novel population of T-cell receptor α/β-bearing thymocytes which predominantly expresses a single Vβ8 gene family. *Nature (Lond.).* 329:251.
17. Tentori, L., D. M. Pardoll, J. C. Zuniga, J. Hu-Li, W. E. Paul, J. A. Bluestone, and A. M. Kruisbeek. 1988. Proliferation and production of IL-2 and B cell stimulatory factor 1/IL-4 in early fetal thymocytes by activation through Thy-1 and CD3. *J. Immunol.* 140:1089.
18. Jenkinson, E. J., R. Kingston, and J. J. T. Owen. 1987. Importance of IL-2 receptors in intra-thymic generation of cells expressing T-cell receptors. *Nature (Lond.).* 329:160.