Preferential Expression of Fibronectin Receptors on Immature Thymocytes

Pina M. Cardarelli, I. Nicholas Crispe,* and Michael D. Pierschbacher

Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037; and
*Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract. Fibronectin-adherent (FNR^+) thymocytes are enriched for immature ($CD4^-8^-$) and large ($CD4^+8^+$) cells, and depleted of mature ($CD4^-8^+$ and $CD4^+8^-$) and nonmature small ($CD4^+8^+$) cells. Among purified $CD4^-8^-$ thymocytes, cells with the surface marker J11d and the IL-2 receptor, which can give rise to all other thymocyte subsets, showed selective attachment to fibronectin. Analysis of FNR^+ thymocytes showed that such cells are greatly enriched for cells in cycle. Additionally, FNR^+ cells expressed low levels of T cell receptor. These results suggest a role for the fibronectin receptor during the early, proliferative phase of thymocyte differentiation. The data suggest that loss of the fibronectin receptor is a hallmark of cells that have become committed either to functional maturation or to programmed cell death.

HEMATOPOIETIC progenitor cells enter the thymus where they undergo proliferation and differentiation before their entry into the peripheral circulation. The cell surface molecules CD4 and CD8 define two subsets of peripheral T lymphocytes. Cells with the $CD4^-8^+$ surface phenotype recognize antigens in association with class I major histocompatibility complex molecules, and cells that are $CD4^+8^-$ recognize antigens plus class II major histocompatibility complex molecules. On thymic lymphocytes, however, the same two surface markers occur in all four possible combinations. The earliest ("immature") thymocytes lack both markers, and can recolonize the thymus of an irradiated host upon adoptive transfer. These $CD4^-8^-$ cells are a minor subpopulation which make up 2–5% of normal thymocytes. Included in the immature subpopulation are large $CD4^+8^+$ blast cells which constitute ~10–15% of the total thymocyte pool. The thymus also contains minor subpopulations of cells that share the $CD4^-8^+$ and $CD4^+8^-$ phenotypes with peripheral T cells, and among these cells are found thymocytes that exhibit "mature" T cell function. The majority of thymocytes however, can be classified as "nonmature." These small cells express the surface phenotype $CD4^+8^+$, and have not been conclusively shown to have either precursor activity or mature T cell function. The role of both $CD4^+8^+$ subsets in T cell differentiation is not known; two main hypotheses suggest that they are either intermediates in differentiation or alternatively, dead-end cells that all die in the thymus leaving no progeny (Ceredig and MacDonald, 1985; Fowlkes and Mathieson, 1985; von Boehmer, 1986; Adkins et al., 1987).

One key role of the thymus in T cell differentiation is the modification of the T cell antigen specificity repertoire, yielding a population of T cells depleted of self-reactive cells. Such a selective effect clearly depends on the expression of antigen-specific receptors, and the rearrangement of T cell receptor genes and the surface expression of their products first occurs within the thymus (Born et al., 1985; Haars et al., 1986). Surface staining for the T cell receptor β chain and the receptor-associated, clonally invariant CD3 complex shows two distinct levels of expression. Low-level expression is found on $CD4^+8^+$ nonmature cells, while high-level expression occurs on the $CD4^+8^-$ and $CD4^-8^+$ subsets which include functionally mature cells (Roehm et al., 1984; Crispe et al., 1987*b*). The transition from low- to high-level expression of T cell receptor may be required for differentiation and selection of mature T cells.

We have previously identified a subpopulation of thymocytes that bind specifically to fibronectin but not to a number of other extracellular matrix molecules (Cardarelli and Pierschbacher, 1986). The interaction between this group of thymocytes and the fibronectin molecule appears to be mediated by the same arginine-glycine-aspartic acid (RGD)[1] sequence (Pierschbacher and Ruoslahti, 1984*a*, *b*) that is recognized by many members of the superfamily of cell surface proteins, collectively known as the integrins, which include the fibronectin receptor, vitronectin receptor, gp IIb/IIIa of platelets, Mac-1, gp 150/95, and LFA-1 (Pytela et al., 1986; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Springer et al., 1987). Fibronectin-adherent (FNR^+) thymocytes express on their surface an integrin-type receptor for fibronectin that is composed of two polypeptides having molecular masses

I. Nicholas Crispe's present address is Division of Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England.

1. *Abbreviations used in this paper*: FNR, fibronectin receptor; FNR^+, fibronectin adherent; FNR^-, fibronectin nonadherent; [^{3}H]TdR, [^{3}H]thymidine; IL-2R, interleukin-2 receptor; RGD, arginine-glycine-aspartic acid.

of 150 and 175 kD under nonreducing conditions (Cardarelli and Pierschbacher, 1987). We have proposed previously that the interaction of thymocytes with fibronectin may be important in directing migration of the developing cells or in stabilizing conjugates between thymocytes and epithelial cells. Fibronectin receptor (FNR) expression may play a role in localizing cells in the thymus. Alternatively, the interaction with this matrix protein may play an instructive or a permissive role in allowing the immature cells to respond to soluble mediators or other mitogenic signals. Indeed, the importance of extracellular matrix molecules in differentiation has been established for other systems (Grobstein, 1975; Gospodarowicz et al., 1978; Pennypacker et al., 1979; Bissell et al., 1982; Watt, 1986; Savagner et al., 1986).

In the present study, we analyze the subset distribution of functional FNR on thymocytes. We find that FNRs are preferentially expressed on the proliferating subpopulations within the $CD4^-8^-$ and $CD4^+8^+$ subsets of thymocytes. Based on the data presented here, it appears that the FNR is important during the early phase of thymocyte development involving cell proliferation and antigen receptor gene rearrangements, and is lost after cell fate has been determined.

Materials and Methods

Reagents

The mAb 3.168 (rat IgM), anti-Lyt-2 (Sarmiento et al., 1980) was grown as ascites, purified by boric acid precipitation, and coupled to FITC by standard methods. The F23.1 antibody (mouse IgG2a), anti-T cell receptor β chain (Staerz et al., 1985) was purified from ascites by protein A chromatography and biotinylated according to the method of Katona et al. (1983). The hybridoma J11d (rat IgM) anti-B cell and cortical thymocyte (Bruce et al., 1981) was grown in vitro, and antibody was concentrated by ammonium sulfate precipitation. Anti-L3T4-phycoerythrin conjugate was purchased from Becton Dickinson & Co. (Mountain View, CA), and Streptavidin-phycoerythrin was from Bio-Meda (Foster City, CA). Antibody RL172.4 (rat IgM) anti-L3T4 (Ceredig et al., 1985) was prepared and used in ascites form. Guinea pig serum was purchased from Pel-Freeze (Rogers, AK). [^{3}H]Thymidine ([^{3}H]TdR; 2.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Fibronectin was prepared from mouse plasma according to Engvall and Ruoslahti (1977). BSA was purchased from Sigma Chemical Co. (St. Louis, MO). Peptides were synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) and purified on a Bio-gel TSK-SP-5PW column (Bio-Rad Laboratories, Richmond, CA) at the La Jolla Cancer Research Foundation.

Thymocyte Attachment to Fibronectin

Thymocytes were isolated by mechanical disruption of young (5-wk) BALB/c thymuses. Attachment to fibronectin-coated polystyrene was assessed after a 2-h incubation at 24°C, as described (Cardarelli and Pierschbacher, 1986). FNR^+ cells were detached by adding 1 mg/ml glycine-arginine-glycine-aspartic acid-serine-proline peptide for 10 min. As described previously (Cardarelli and Pierschbacher, 1987), this method detaches all adhering thymocytes. Fibroblastic cells and macrophages were resistant to this brief detachment period and did not appear in our analysis. The cells were then washed and analyzed.

Incorporation of [^{3}H]TdR

FNR^+, FNR^-, or unselected thymocytes (1×10^6 cells per well) were cultured in microwells (96-well, Linbro, Flow Laboratories, Hamden, CT) containing 1 μCi/well of [^{3}H]TdR. Periodically, the cpm in each fraction was assessed. Proliferation was assessed by the incorporation of [^{3}H]TdR (1 μCi/well). The mean ± SEM of six wells is expressed.

Surface Staining

Aliquots of 10^5–10^6 unselected or selected thymocytes were suspended in 50 μl of Hanks' balanced salt solution (HBSS) containing 2.5% FCS (Flow Laboratories, Inc., McLean, VA). Staining reagents were added to predetermined saturating concentrations, and cells were stained on ice for 45 min. (This time was required for saturating F23.1 staining, although supraoptimal for the other stains). Each aliquot was diluted in 3 ml of HBSS plus 2.5% FCS, cells were pelleted and resuspended in either 0.5 ml of 1% paraformaldehyde fixative or 50 μl of second-layer staining reagent. After two-step staining, cells were again washed and fixed. Flow cytometric analysis was performed on a FACS IV (Becton Dickinson & Co.) using the Consort 30 data analysis package. Intact cells were demarcated from dead cells and debris using forward and sideways light scatter, and 10,000 particles were analyzed for one- or two-color fluorescence. Results are presented either as single-color histograms or as contour plots using logarithmic contour intervals (3, 9, 27, and 81 cells per pixel). For F23.1 staining, the division into TCR^-, TCR^{lo}, and TCR^{hi} thymocytes was chosen on unselected thymocytes where three discrete staining intensities were obtained. These gates were maintained for analysis of FNR^+ and fibronectin-nonadherent (FNR^-) cells.

Cell Cycle Analysis

Cells were classified into the cell cycle stages G_0, G_1, S, or G_2/M based on flow cytometric measurement of DNA and RNA content according to the method of Darzynkiewicz et al. (1980). Briefly, cells were permeabilized in a buffer containing 0.1% Triton and 10^{-4} M EDTA, then acridine orange (Polysciences Inc., Warrington, PA) was added to a final concentration of 10 μg/ml. After 2–5 min of equilibration, cells were analyzed for green DNA fluorescence (using 515–530-nm-band pass filters) and red RNA fluorescence (using a 630-nm-long pass filter). Contour plots were used to establish markers that separated cells with baseline RNA and DNA levels (G_0); cells with baseline DNA but more RNA (G_1); cells with intermediate DNA levels (S); and cells with twofold baseline DNA levels (G_2/M). Dead cells and debris were excluded from analysis as in surface fluorescence measurements.

Isolation of $CD4^-8^-$ Thymocytes

Cells were suspended at 2–4 × 10^7/ml in HBSS plus 2.5% FCS. The cytotoxic antibodies 3.168 and RL.172.4 were added to saturating concentrations. After 30 min on ice, guinea pig serum was added as a source of complement, and cell suspensions were warmed to 37°C to allow lysis. Two complete killing cycles were performed to give $CD4^-8^-$ thymocytes, which were routinely ~99% pure on restaining and two-color FACS analysis.

Separation of Cells by Velocity Sedimentation

Thymocytes, 1×10^7 cells/ml in 40 ml of 0.25% BSA, were isolated and separated by size using the technique of velocity sedimentation (Miller and Phillips, 1969; Miller, 1973). Fractions of large and small cells were collected and pooled separately, while the fractions that contained both large and small cells (constituting ≤10% of the cells) were discarded. The cells were analyzed for [^{3}H]TdR incorporation in overnight culture or stained for cell surface markers CD4 and CD8.

Results

FNR^+ Thymocytes Are Mostly $CD4^-8^-$ and $CD4^+8^+$

In preliminary experiments, we found the cell surface phenotype of unselected BALB/c thymocytes to be mostly (78%) $CD4^+8^+$, with a minor (3%) $CD4^-8^-$ subpopulation and clear-cut $CD4^+8^-$ (13%) and $CD4^-8^+$ (5%) subpopulations as assessed by two-color FACS analysis. In these FACS analyses we also saw a distinct $CD4^-8^{dull}$ subset, very infrequent (<2%) in unselected thymocytes but more prominent in the subpopulations selected by FNR-mediated adhesion. In the analyses presented here we have chosen to regard these cells as distinct from "mature" $CD4^-8^+$ thymocytes and we

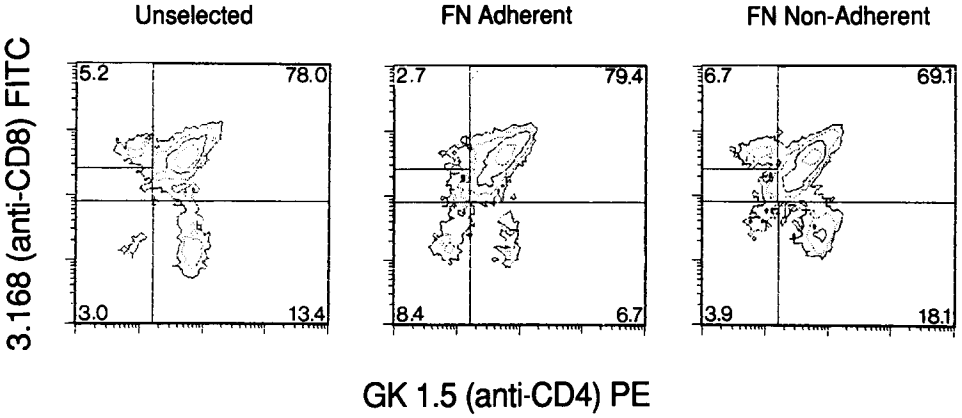

Figure 1. Cell surface phenotype of FNR$^+$ thymocytes. Unselected, FNR$^+$, and FNR$^-$ thymocytes were collected and stained by two-color FACS analysis using antibodies against CD4 and CD8. The percentage of positive cells in each quadrant are indicated.

will discuss them separately. Fig. 1 shows that adhesion to fibronectin enriches for immature, "double-negative" (CD4$^-$8$^-$) cells. The FNR$^+$ cells were also slightly enriched for a subpopulation of cells that express both CD4 and CD8 ("double-positive" cells) and depleted of thymocytes that have the more mature "single-positive" phenotypes, CD4$^+$8$^-$ or CD4$^-$8$^+$. Transfer of FNR$^-$ cells to a second fibronectin-coated dish showed that the FNR$^+$ cells had been quantitatively removed in the first incubation. It remains to be determined whether a subpopulation of FNR$^+$ single-positive cells exists, or whether this is a contamination due to the limits of the panning technique used. The antibodies currently available (anti-human fibroblast fibronectin receptor) stain mouse cells poorly by immunofluorescence, so flow cytometric reanalysis of the selected FNR$^+$ and FNR$^-$ cells is not possible. Overall, fibronectin adhesion selected for cells with a more immature phenotype and selected against mature thymocytes. To verify that FNR$^+$ cells were depleted of mature thymocytes, we also assessed expression of T cell antigen receptor.

Expression of T Cell Antigen Receptor β Chain (F23.1) on FNR$^+$ Thymocytes

The antibody F23.1 recognizes an epitope on the β chain of the T cell antigen receptor (Epstein et al., 1985; Sim and Augustin, 1985). High-level β chain expression appears predominantly on CD4$^+$8$^-$ and CD4$^-$8$^+$ cells, and expression at low levels is mainly on a subpopulation of CD4$^+$8$^+$ cells (Roehm et al., 1984; Crispe et al., 1987*b*). Therefore, FNR$^+$ cells from total thymus were stained with the F23.1 antibody. When compared to unselected thymocytes, high-level (F23.1^{hi}) expression on the FNR$^+$ thymocytes was significantly reduced (Fig. 2) with 1.7% expressing high levels of F23.1 as compared to 5.6% of unselected thymocytes. In contrast, the FNR$^+$ subpopulation contained a proportion of F23.1^{lo} cells similar to that of unselected thymocytes. These data show that fibronectin adherence is associated with thymocyte subsets that are TCR$^-$ or TCR^{lo}. Since FNR$^+$ cells are enriched for immature cells by several phenotypic criteria, we tested them for their proliferative capability.

Cell Cycle Analysis of FNR$^+$ Thymocytes

It has been reported that ~15% of all young adult thymocytes are proliferating with an average cell cycle time of 9 h (Metcalf, 1966; Rothenberg and Lugo, 1985). Thymic lymphoblasts fall mainly into two subpopulations, CD4$^-$8$^-$ and CD4$^+$8$^+$, and fibronectin adherence selects for these thymocyte subsets. Because this suggests an association between FNR expression and proliferation, we carried out cell cycle analysis using acridine orange staining on the total and FNR$^+$ cells (Table I). Among unselected thymocytes, 83% were in G_0. In contrast, FNR$^+$ cells were much more frequently in cycle. Thus, there is a strong bias towards proliferation in the FNR$^+$ thymocyte population.

[^{3}H]TdR Incorporation by FNR$^+$ Thymocytes

Because enrichment of proliferating cells was obtained by panning on fibronectin, we next asked whether adherence to fibronectin directly delivers a mitogenic signal. To address

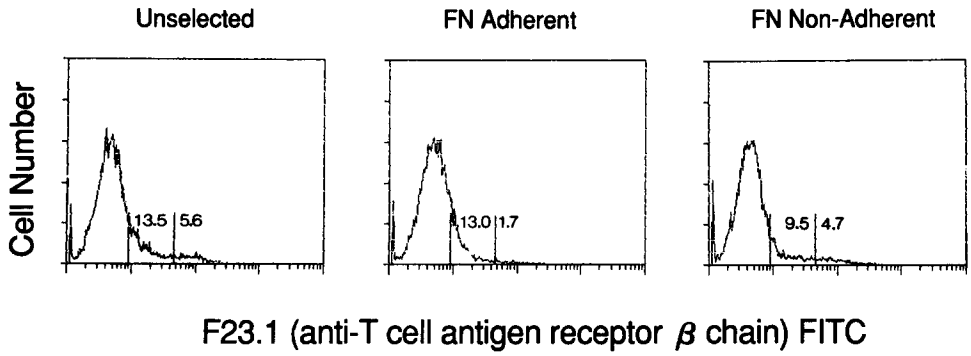

Figure 2. Expression of F23.1 (anti-T cell antigen receptor β chain) on FNR$^+$ thymocytes. Unselected, FNR$^+$, and FNR$^-$ thymocytes were collected and stained by single-color FACS. The number of cells that are TCR$^-$, TCR^{lo}, and TCR^{hi} are indicated.

Table I. Cell Cycle Analysis of FNR⁺ Thymocytes

Thymocytes	Cell cycle stage (AO): % of cells			
	G_0	G_1	S	G_2/M
Unselected	82.8	4.3	5.1	5.0
FNR⁺	55.7	12.6	12.1	15.6

Table II. Cell Cycle Analysis of FNR⁺ CD4⁻8⁻ Thymocytes

CD4⁻8⁻Thymocytes	Cell cycle stage (AO): % of cells			
	G_0	G_1	S	G_2/M
Unselected	61.3	13.2	14.4	9.6
FNR⁺	48.8	16.4	19.0	13.6

this, the FNR⁺, FNR⁻, and unselected thymocytes were first incubated for various time intervals in the presence of [³H]TdR, and incorporation was assessed (Fig. 3). At all time points studied, [³H]TdR incorporation was significantly higher in the FNR⁺ subpopulation. Even after 24 h, thymidine incorporation was threefold higher in the FNR⁺ group when compared to FNR⁻ cells. Neither addition of recombinant interleukin-2 nor culturing thymocytes on fibronectin-coated substrates had an effect on the proliferative capacity in all three groups (data not shown). Additionally, by microscopic evaluation the number of cells present after overnight incubation was greater in the FNR⁺ fraction. These data are in agreement with the cell cycle analysis data which showed that FNR⁺ thymocytes are enriched for proliferating cells. Furthermore, fibronectin itself does not appear to provide a mitogenic signal.

Attachment of CD4⁻8⁻ Thymocytes to Fibronectin

Since FNR⁺ thymocytes are enriched for cells with the CD4⁻8⁻ phenotype (see Fig. 1), purified double-negative cells were made by two cycles of antibody and complement treatment, and these cells were 99% pure CD4⁻8⁻ cells as judged by two-color FACS analysis (data not shown). These double-negative cells were then assessed for their ability to bind to fibronectin-coated dishes. An estimated 20 ± 5% of CD4⁻8⁻ cells bound specifically to fibronectin-coated dishes (compared to 10% of total thymocytes) (Cardarelli and Pierschbacher, 1986). After detachment of the FNR⁺, double-negative cells with the RGD-containing peptide, the cells were stained with antibodies directed against interleukin-2 receptor (IL-2R) and J11d, which define functionally distinct subsets of CD4⁻8⁻ cells (Crispe et al., 1987*a*; Shimonkevitz et al., 1987). We found that 74% of these FNR⁺ CD4⁻8⁻ cells were positive for J11d and 36% carried IL-2R on their surface (Fig. 4), whereas the FNR⁻ subset was 45% positive for J11d and 22% positive for IL-2R. Thus, the fibronectin panning enriched for cells that express J11d and IL-2R.

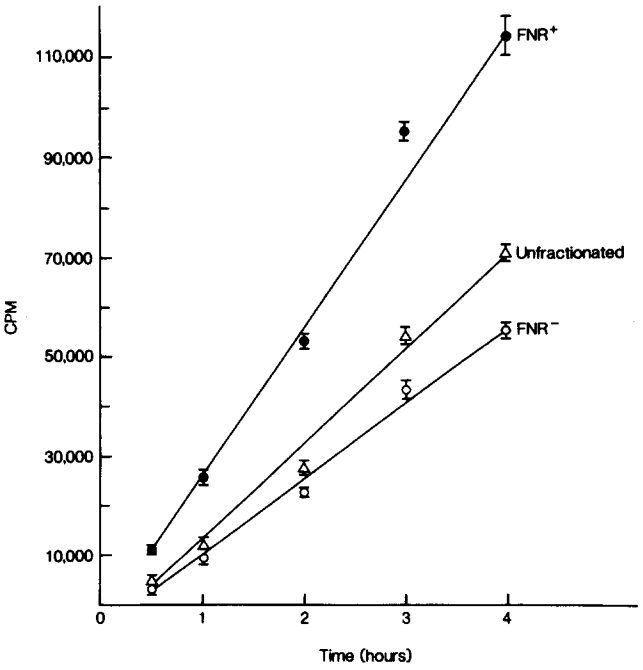

Figure 3. Time-course of [³H]TdR incorporation of FNR⁺ and FNR⁻ thymocytes. FNR⁺ thymocytes, FNR⁻ thymocytes, or unfractionated thymocytes (1 × 10^6 cells per well) were incubated for 30 min, or 1, 2, 3, or 4 h in the presence of 1 μCi of [³H]TdR. Each point represents the mean ± SEM, n = 4. The correlation coefficient for each line was >0.99.

Cell Cycle Analysis of FNR⁺, CD4⁻8⁻ Thymocytes

Table II shows that, based on acridine orange cell cycle analysis, CD4⁻8⁻ cells are more often in cycle than are total thymocytes (Table I). Selection of CD4⁻8⁻ cells by virtue of their adhesion to fibronectin enriches further for cells in G_1, S, and G_2/M. This enrichment could not account for the increase obtained from thymidine uptake data and cell cycle analysis of total FNR⁺ thymocytes, because CD4⁻8⁻ make up such a small proportion of total thymocytes. This fact, together with the observation that total FNR⁺ thymocytes are not depleted of CD4⁺8⁺ cells, strongly suggests that FNR⁺ CD4⁺8⁺ cells also contain a subpopulation of blast cells.

The preceding data on unselected thymocytes and isolated CD4⁻8⁻ cells indicate that FNR⁺ cells are greatly enriched for cells in cycle. To determine whether FNR expression is associated with all thymic blast cells or only with subpopulations of blast cells, it was necessary to examine blast cells in isolation.

Cell Surface Phenotype of FNR⁺ Blast Cells

Large thymocytes were separated from small cells by velocity sedimentation using a BSA density gradient (Miller and Phillips, 1969; Miller, 1973). In the experiment shown, 17% of the thymocytes were large blast cells. Two-color FACS analysis of the isolated blast cells (Fig. 5) showed that all four major subpopulations of thymocytes were represented, but CD4⁻8⁻ cells were highly enriched (14%). Both "single-positive" populations were present and there was a significant proportion (4%) of CD4⁻8^{dull} cells. Of the total isolated large cells, ~50% attached to fibronectin; this reflects extensive enrichment for FNR⁺ cells by this procedure (Table III). The large cells were greatly enriched for cells in cycle, as judged by [³H]TdR incorporation; and both FNR⁺ and

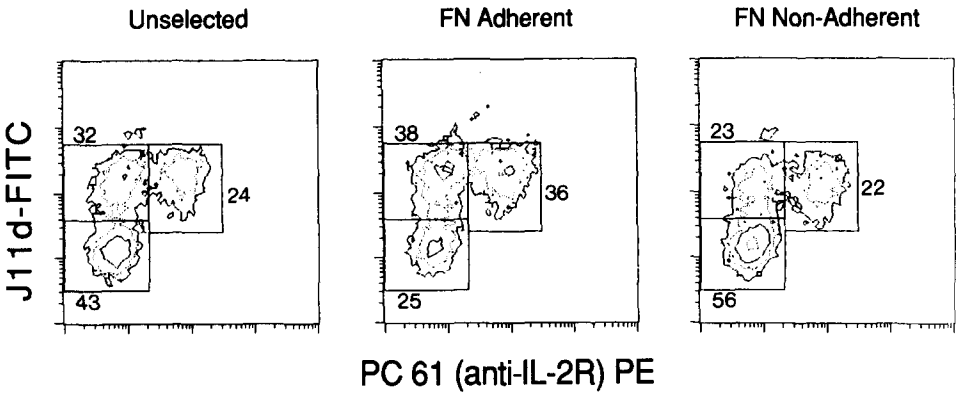

Figure 4. Cell surface phenotype of FNR$^+$ CD4$^-$8$^-$ thymocytes. Double-negative (CD4$^-$8$^-$) thymocytes were prepared by two cycles of antibody and complement. The purified CD4$^-$8$^-$ cells that were FNR$^+$, FNR$^-$, or unselected were stained and analyzed by two-color FACS using the antibodies J11d and PC61 (anti-IL-2R). The percentage of cells that were positive for each antibody is shown.

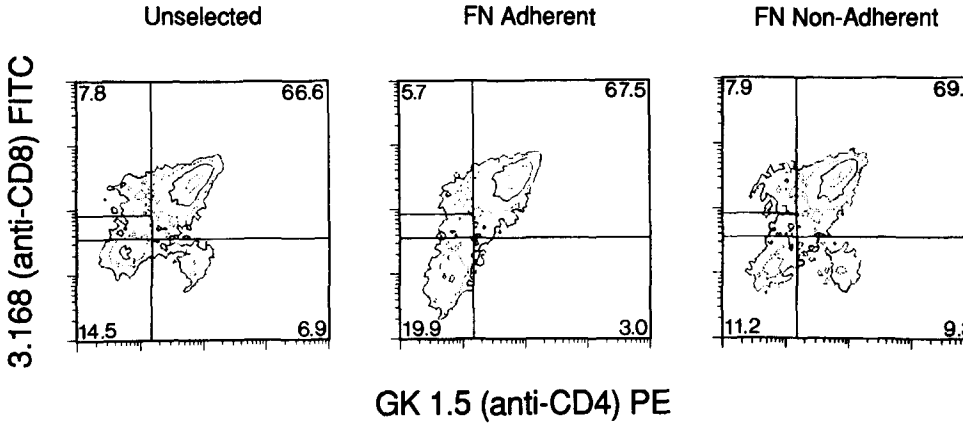

Figure 5. Cell surface phenotype of FNR$^+$ blast cells. Large thymocytes were separated from small and intermediate size cells using a BSA density gradient. Blast cells that were unselected, FNR$^+$, and FNR$^-$ were collected and stained by two-color FACS analysis using antibodies directed against CD4 and CD8. The percentage of cells that were positive for the antibodies are indicated in each quadrant.

FNR$^-$ blast cells incorporated thymidine equally (data not shown). In contrast, the isolated small cells did not incorporate [^{3}H]TdR, nor did they attach well to fibronectin. This suggests that while not all blast cells are FNR$^+$, most FNR$^+$ cells are blast cells (Table III). In comparison to the total blast cells, however, the isolated FNR$^+$ blast cells were mostly CD4$^-$8$^-$ and CD4$^+$8$^+$, and CD4$^+$8$^-$ cells were strikingly absent (the boundaries used to define the subpopulations scored 3% of cells as CD4$^+$8$^-$ in this analysis, but the contour plot shows that most of this is overlap from other subsets and bona fide CD4$^+$8$^-$ cells were much rarer). Both CD4$^-$8$^+$ and CD4$^-$8^{dull} cells were present, although the former were slightly depleted (6%). In the FNR$^-$ blast cells, CD4$^+$8$^-$ cells were prominent (9%) and CD4$^-$8$^+$ cells (8%) were a little more prominent than CD4$^-$8^{dull} cells (3%). Thus, the selection of blast cells on the basis of FNR expression had only a subtle effect on CD4$^-$8$^+$ cells, but completely altered the proportion of CD4$^+$8$^-$ cells. This distinction between the two categories of "single-positive" thymocytes has parallels in a number of other phenotypic and functional characteristics (see Discussion).

Discussion

We have proposed that the extracellular matrix protein, fibronectin, plays a role in T cell differentiation (Cardarelli and Pierschbacher, 1986, 1987). In this study, we have examined the distribution of FNR on subpopulations of mouse thymocytes. RGD-dependent fibronectin-binding activity is found mainly on immature thymocytes with the phenotypes CD4$^-$8$^-$ and CD4$^+$8$^+$, while it is rare among mature cells of the CD4$^-$8$^+$ and CD4$^+$8$^-$ phenotypes. In contrast, thymocytes with high surface density of antigen receptors, detected by the F23.1 antibody against a family of V_β gene products, segregate in the FNR$^-$ subpopulation.

Previous studies have suggested that most proliferative activity in the thymus occurs in the CD4$^-$8$^-$ and CD4$^+$8$^+$ thymocytes subsets (Scollay et al., 1984) where we have detected FNR$^+$ cells. By using acridine orange cell cycle analysis as well as [^{3}H]TdR incorporation, we found that FNR$^+$ thymocytes were indeed greatly enriched for cells in cycle. Thymidine incorporation of FNR$^+$ cells was not affected by any of the substrates on which they were cultured even in the presence of exogenous interleukin-2; this suggests that fibronectin itself was not delivering a mitogenic signal to thymocytes during overnight culture. Gospodarowicz et al. (1986) have shown that the ability of fibroblast growth factor to promote cell proliferation correlates with its ability to modulate synthesis of extracellular matrix components such as fibronectin. They propose that the effect of the matrix on cell proliferation may be a permissive one resulting in an in-

Table III. Adhesion of Thymocyte Blast Cells to Fibronectin

	FNR$^+$	FNR$^-$	Adherent cells*
	n	*n*	%
Blast cells	6.55 × 10^6	6.85 × 10^6	48.9
Small cells	0.70 × 10^6	47.5 × 10^6	1.4

* Numerical addition of FNR$^+$ blast cells and small cells shows that ~11.7% of the cells attach to fibronectin, which is in good agreement with what we find when unselected thymocytes are fractionated by panning on fibronectin.

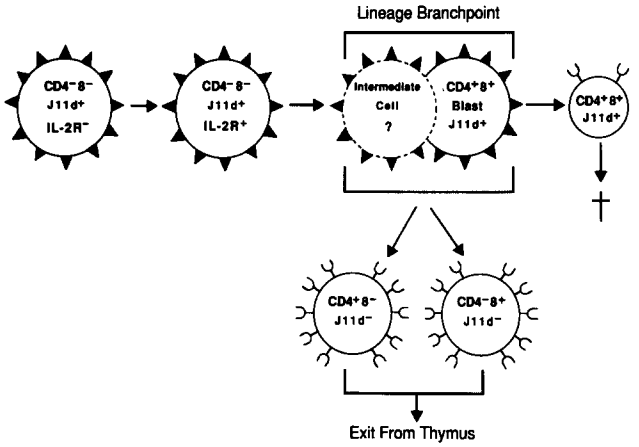

Figure 6. Model of FNR expression during thymocyte differentiation. The model proposes that FNRs (▲) are expressed on early $CD4^-8^-$ cells that are $J11d^+$. After transiently expressing the IL-2R, the cells reach a branch point at which their developmental fate is determined. The cell labeled *Intermediate cell?* indicates that it is still not clear whether the functionally mature cells and the small $CD4^+8^+$ cells both derived from $CD4^+8^+$ blasts or from some common precursor that is not $CD4^+8^+$. We propose that the cells of this branch point are FNR^+ and lack high-level expression of T cell antigen receptor (Y). These cells can become small $CD4^+8^+$ cells that are believed to be committed to cell death (†). Alternatively, they can give rise to $CD4^+8^-$ or $CD4^-8^+$ cells which lack FNR and express high levels of T cell antigen receptor. We propose that the loss of both FNR and J11d is associated with maturation of functional T cells that are then competent to leave the thymus.

creased cell sensitivity to plasma factors. Whether FNR^+ thymocytes are more sensitive to soluble factors within the thymus has yet to be determined.

The earliest thymocytes are $CD4^-8^-$ (Ceredig et al., 1983), and these cells appear to give rise to all thymocyte subsets in vitro (Fowlkes et al., 1984; Kisielow et al., 1984) and in vivo (Fowlkes et al., 1985). However, the $CD4^-8^-$ subpopulation of thymocytes is itself heterogeneous (Scollay et al., 1984; Fowlkes and Mathieson, 1985; Ceredig et al., 1985; Raulet, 1985; Scollay and Shortman, 1985). Recent studies of the recolonization potential of some of these subsets show that early T cells express the marker J11d (Crispe et al., 1987*a*) but lack the IL-2R, while their immediate differentiation products are $IL\text{-}2R^+$ (Shimonkevitz et al., 1987). Because we found a greatly increased proportion of $CD4^-8^-$ cells among FNR^+ thymocytes, we correlated FNR expression with the subset markers J11d and IL-2R on isolated $CD4^-8^-$ thymocytes. The $FNR^+CD4^-8^-$ cells were enriched for the $J11d^+IL\text{-}2R^-$ and $J11d^+$ $IL\text{-}2R^+$ subsets, both of which are early stages in T cell differentiation. On the other hand, the $FNR^-CD4^-8^-$ cells were enriched for the $J11d^-IL\text{-}2R^-$ subset, a cell population which lacks precursor function in fetal organ culture and in vivo (Crispe et al., 1987*a*), lacks voltage-gated K^+ channels (McKinnon and Ceredig, 1986), and expresses antigen receptors using the $V_\beta 8$ gene family on a uniquely high proportion of cells (>50%) (Budd et al., 1987; Fowlkes et al., 1987). In parallel with the surface staining, acridine orange cell cycle analysis showed that the FNR^+ subpopulation of $CD4^-8^-$ thymocytes was enriched for cells in cycle.

To determine whether FNR expression was associated with cell size per se or with particular subpopulations of blast cells, we prepared a thymocyte fraction greatly enriched for large cells using velocity gradient sedimentation, and then separated the cells into FNR^+ and FNR^-. The blast cells were enriched for $CD4^-8^-$ cells, but all of the other cell populations were well represented (including the $CD4^-8^+$ and $CD4^+8^-$). Strikingly, the FNR^+ subset of the blasts were essentially devoid of $CD4^+8^-$ cells, and was composed mainly of $CD4^-8^-$ and $CD4^+8^+$ cells.

Previous work suggests an asymmetry between $CD4^-8^+$ and $CD4^+8^-$ thymocytes. Only the $CD4^-8^+$ contain functionally distinct subsets defined by the progenitor and cortical thymocyte marker J11d; the $J11d^+CD4^-8^+$ cells are functionally incompetent while the $J11d^-CD4^-8^+$ cells share the activation characteristics and cytotoxic T cell precursor activity of peripheral $CD8^+$ T cells (Crispe and Bevan, 1987). In a three-color flow cytometric analysis of CD3 expression, $CD4^-8^+$ but not $CD4^+8^-$ thymocytes contained distinct $CD3^+$ and $CD3^{dull/-}$ subpopulations (Bluestone et al., 1987). Similarly, the $J11d^+CD4^-8^+$ cells lack TCR β chains (Crispe et al., 1987*b*). We now find another sharp distinction; $CD4^-8^+$ blast cells can be readily divided into FNR^+ and FNR^- cells although the FNR^+ cells express lower overall levels of CD8. In contrast, no $FNR^+CD4^+8^-$ blast cells were found.

Fibronectin has been shown to promote cell migration, particularly during embryonic development (Thiery et al., 1985). Either RGD-containing peptides or antibodies to the integrin complex inhibit embryonal cell migration (Boucaut et al., 1984; Bronner-Fraser, 1985). Additionally, the expression of fibronectin receptors on erythroid and myeloid progenitor cells have been postulated to be involved in maintaining the cells anchored in the bone marrow until their release at which time they lose their ability to bind to fibronectin (Patel and Lodish, 1984; Patel et al., 1985; Giancotti et al., 1986; Patel and Lodish, 1986). In an analogous manner, loss of FNR on the developing thymocyte may be essential for the release of mature T cells from the thymus. However, this hypothesis should be approached with caution, as small $CD4^+8^+$ thymocytes which die intrathymically do not express FNR. We have found, however, that resting peripheral blood lymphocytes, both T and B cells, do not attach to fibronectin (unpublished observation). Our studies also show that many FNR^+ thymocytes express TCR at a low level, indicating that at least the majority of FNR^+ cells are cortical and at early stages of development (Kyewski et al., 1984; Farr et al., 1985; Kingston et al., 1985). However, the overlap in time between the first expression of antigen-specific molecules and loss of the fibronectin receptor might be explained by the need for extensive modification of the T cell repertoire before cells attain functional competence.

In the present study, we have found FNR expressed preferentially on early ($J11d^+CD4^-8^-$) precursor cells and on $CD4^+8^+$ blast cells, and selectively lacking on functionally mature ($CD4^-8^+$ and $CD4^+8^-$) cells and cells committed to intrathymic death (small $CD4^+8^+$ cells). On this basis we propose that FNRs may play a role in early differentiation events before lineage commitment occurs. Then, after some critical branch point(s) in the differentiation pathway, the cells down-regulate the FNR, and those which are destined to exit as functional T cells also upregulate the TCR (Fig. 6).

Although the exact surface phenotype of the selectable cells remains unknown, it is probable, based on the data presented here, that they express FNRs. Such receptors would provide a means of obtaining purified populations of these selectable cells. In addition, an understanding of the adhesive interactions that take place in the thymus may be vital in our understanding of the events that dictate cell commitment.

We wish to thank Drs. E. Ruoslahti, W. Lernhardt, and M. J. Bevan for their comments on the manuscript.

This work was supported by Cancer Center Support grant CA 30199 and U. S. Public Health Service grant CA 38352 to M. D. Pierschbacher and grants AI 19335 and CA 25803 to Dr. Michael J. Bevan. P. M. Cardarelli was supported by postdoctoral fellowship HL 07194-09 from the National Institutes of Health, and I. N. Crispe was supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer fund (DRG-774).

Received for publication 20 November 1987, and in revised form 22 February 1988.

References

Adkins, B., C. Mueller, C. Y. Okada, R. A. Reichert, I. L. Weissman, and G. J. Spangrude. 1987. Early events in T-cell maturation. *Annu. Rev. Immunol.* 5:325–365.

Bissell, M. J., H. G. Hall, and G. Parry. 1982. How does the extracellular matrix direct gene expression? *J. Theor. Biol.* 99:31–68.

Bluestone, J. A., D. M. Pardoll, S. O. Sharrow, and B. J. Fowlkes. 1987. Ontogeny, phenotype and functional characterization of murine thymocytes possessing T3-associated T cell receptor structures. *Nature (Lond.).* 326: 82–84.

Born, W., J. Yague, E. Palmer, J. Kappler, and P. Marrack. 1985. Rearrangement of T-cell receptor β-chain genes during T-cell development. *Proc. Natl. Acad. Sci. USA.* 82:2925–2929.

Boucaut, J.-C., T. Darribere, T. J. Poole, H. Aoyama, K. M. Yamada, and J.-P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos, and neural crest cell migration in avian embryos. *J. Cell Biol.* 99:1822–1830.

Bronner-Fraser, M. 1985. Alteration in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* 101:610–617.

Bruce, J., F. W. Symington, T. J. McKearn, and J. Sprent. 1981. A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* 127: 2496–2501.

Budd, R. C., G. C. Miescher, R. C. Hare, R. K. Lees, C. Bron, and H. R. MacDonald. 1987. Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes. *J. Exp. Med.* 166: 577–582.

Cardarelli, P. M., and M. D. Pierschbacher. 1986. T-lymphocyte differentiation and the extracellular matrix: identification of a thymocyte subset that attaches specifically to fibronectin. *Proc. Natl. Acad. Sci. USA.* 83:2647–2651.

Cardarelli, P. M., and M. D. Pierschbacher. 1987. Identification of fibronectin receptors on T-lymphocytes. *J. Cell Biol.* 105:499–506.

Ceredig, R., and H. R. MacDonald. 1985. Intrathymic differentiation: some unanswered questions. *Surv. Immunol. Res.* 4:87–95.

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.).* 314:98–100.

Ceredig, R., H. R. MacDonald, and E. J. Jenkinson. 1983. Flow microfluorometric analysis of mouse thymus development in vivo and in vitro. *Eur. J. Immunol.* 13:185–190.

Crispe, I. N., and M. J. Bevan. 1987. Expression and functional significance of the J11d marker on mouse thymocytes. *J. Immunol.* 138:2013–2018.

Crispe, I. N., M. Moore, L. A. Husmann, L. F. Smith, M. J. Bevan, and R. P. Shimonkevitz. 1987*a*. Differentiation potential of subsets of $CD4^-8^-$ thymocytes. *Nature (Lond.).* 329:336–339.

Crispe, I. N., R. P. Shimonkevitz, L. A. Husmann, J. Kimura, and J. P. Allison. 1987*b*. Expression of T cell antigen receptor beta chains on subsets of mouse thymocytes: analysis by three-color flow cytometry. *J. Immunol.* 139:3585–3589.

Darzynkiewicz, Z., T. Sharpless, L. Staiano-Coico, and M. R. Melamed. 1980. Subcompartments of the G1 phase of cell cycle detected by flow cytometry. *Proc. Natl. Acad. Sci. USA.* 77:6696–6699.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

Epstein, R., N. Roehm, P. Marrack, J. Kappler, M. Davies, S. Hedrick, and M. Cohn. 1985. Genetic markers of the antigen-specific T cell receptor locus. *J. Exp. Med.* 161:1219–1224.

Farr, A. G., S. K. Anderson, P. Marrack, and J. Kappler. 1985. Expression of antigen-specific, major histocompatibility complex-restricted receptors by cortical and medullary thymocytes in situ. *Cell.* 43:543–550.

Fowlkes, B. J., and B. J. Mathieson. 1985. Intrathymic differentiation: thymocyte heterogeneity and the characterization of early T-cell precursors. *Surv. Immunol. Res.* 4:96–109.

Fowlkes, B. J., L. Edison, B. Mathieson, and T. M. Chused. 1984. Differentiation in vitro of an adult precursor thymocyte. *In* Regulation of the Immune System. H. Cantor, L. Chess, and E. Sercarz, editors. Alan R. Liss Inc., New York. 275–293.

Fowlkes, B. J., L. Edison, B. J. Mathieson, and T. M. Chused. 1985. Early T lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. *J. Exp. Med.* 162:802–822.

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 alpha beta-bearing thymocytes which predominantly express a single V_β gene family. *Nature (Lond.).* 329:251–254.

Giancotti, F. G., P. M. Comoglio, and G. Tarone. 1986. Fibronectin-plasma membrane interaction in the adhesion of hemopoietic cells. *J. Cell Biol.* 103:429–437.

Gospodarowicz, D., G. Greenburg, and C. R. Birdwell. 1978. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res.* 38:4155–4171.

Gospodarowicz, D., G. Neufeld, and L. Schweigerer. 1986. Molecular and biological characterization of fibroblast growth factor: an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. *Cell Differen.* 19:1–17.

Grobstein, C. 1975. Extracellular Matrix Influences on Gene Expression. H. C. Slavkin and R. C. Grelich, editors. Academic Press, Inc., New York. 833 pp.

Haars, R., M. Kronenberg, M. W. Gallatin, I. L. Weissman, F. L. Owen, and L. Hood. 1986. Rearrangement of T cell antigen receptor and gamma genes during thymic development. *J. Exp. Med.* 164:1–24.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

Katona, I. M., J. F. Urban, Jr., I. Scher, C. Kanellopoulos-Langevin, and F. D. Finkelman. 1983. Induction of IgE response in mice by Nippostrongylus brasiliensis: characterization of lymphoid cells with intracytoplasmic or surface IgE. *J. Immunol.* 130:350–356.

Kingston, R., E. J. Jenkinson, and J. J. T. Owen. 1985. A single stem cell can recolonize an embryonic thymus producing phenotypically distinct T-cell populations. *Nature (Lond.).* 317:811–813.

Kisielow, P., W. Leiserson, and H. von Boehmer. 1984. Differentiation of thymocytes in fetal organ culture: analysis of phenotypic changes accompanying the appearance of cytolytic and interleukin-2 producing cells. *J. Immunol.* 133:1117–1123.

Kyewski, B. A., M. Travis, and H. S. Kaplan. 1984. Intrathymic lymphopoiesis: stromal cell associated proliferation of T cell is independent of lymphocyte genotype. *J. Immunol.* 133:1111–1116.

McKinnon, D., and R. Ceredig. 1986. Changes in the expression of potassium channels during mouse T cell development. *J. Exp. Med.* 164:1846–1861.

Metcalf, D. 1966. Structure of the thymus. *In* The Thymus. Recent Results in Cancer Research. D. Metcalf, editor. Springer-Verlag New York Inc., Berlin. 1–17.

Miller, R. G. 1973. Separation of cells by velocity sedimentation. *In* New Techniques in Biophysics and Cell Biology. R. Pain and B. Smith, editors. John Wiley & Sons, London. 87–112.

Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* 73:191–201.

Patel, V. P., and H. F. Lodish. 1984. Loss of adhesion of murine erythroleukemia cells to fibronectin during erythroid differentiation. *Science (Wash. DC).* 224:996–998.

Patel, V. P., and H. F. Lodish. 1986. The fibronectin receptor on murine erythroleukemia cells: characterization and developmental regulation. *J. Cell Biol.* 102:449–456.

Patel, V. P., A. Ciechanover, O. Platt, and H. F. Lodish. 1985. Mammalian reticulocytes lose adhesion to fibronectin during maturation to erythrocytes. *Proc. Natl. Acad. Sci. USA.* 82:440–444.

Pennypacker, J. P., J. R. Hassell, K. M. Yamada, and R. M. Pratt. 1979. The influence of an adhesive cell surface protein on chondrogenic expression in vitro. *Exp. Cell Res.* 121:411–415.

Pierschbacher, M. D., and E. Ruoslahti. 1984*a*. The cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.

Pierschbacher, M. D., and E. Ruoslahti. 1984*b*. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc. Natl. Acad. Sci. USA.* 81:5985–5988.

Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa is a member of a family of arg-gly-asp-specific adhesion receptors. *Science (Wash. DC).* 231:1559–1562.

Raulet, D. H. 1985. Expression and function of interleukin-2 receptors on immature thymocytes. *Nature (Lond.).* 314:101–103.

Roehm, N., L. Herron, J. Cambier, D. DiGuisto, 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.* 38:577–584.

Rothenberg, E., and J. P. Lugo. 1985. Differentiation and cell division in the mammalian thymus. *Dev. Biol.* 112:1–17.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC).* 238:491–497.

Sarmiento, M., A. L. Glasbrook, and F. W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement. *J. Immunol.* 125:2665–2672.

Savagner, P., B. A. Imhof, K. M. Yamada, and J.-P. Thiery. 1986. Homing of hemopoietic precursor cells to the embryonic thymus: characterization of an invasive mechanism induced by chemotactic peptides. *J. Cell Biol.* 103: 2715–2727.

Scollay, R., and K. Shortman. 1985. Identification of early stages of T lymphocyte development in the thymus cortex and medulla. *J. Immunol.* 134:3632–3642.

Scollay, R., P. Bartlett, and K. Shortman. 1984. T cell development in the adult murine thymus: changes in the expression of the surface antigens Ly 2, L3T4 and B2A2 during development from early precursor cells to emigrants. *Immunol. Rev.* 82:79–103.

Shimonkevitz, R. P., L. A. Husmann, M. J. Bevan, and I. N. Crispe. 1987. Expression of the interleukin-2 receptor precedes the differentation of immature thymocytes. *Nature (Lond.).* 329:157–159.

Sim, G. K., and A. A. Augustin. 1985. V_β gene polymorphism and a major polyclonal T cell receptor idiotype. *Cell.* 42:89–92.

Springer, T. A., M. L. Dustin, T. K. Kishimoto, and S. D. Marlin. 1987. The lymphocyte function-associated LFA-1, CD2 and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu. Rev. Immunol.* 5:223–252.

Staerz, U. D., H. G. Rammenssee, 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.* 134:3994–4000.

Thiery, J.-P., J. L. Duband, and G. C. Tucker. 1985. Cell migration in the vertebrate embryo: role of cell adhesion and tissue environment in pattern formation. *Annu. Rev. Cell Biol.* 1:91–113.

von Boehmer, H. 1986. The selection of the alpha, beta heterodimeric T-cell receptor for antigen. *Immunol. Today.* 7:333–336.

Watt, F. M. 1986. The extracellular matrix and cell shape. *Trends Biochem. Sci.* 11:482–485.