

IDENTIFICATION AND CLONING OF A PRETHYMIC
PRECURSOR T LYMPHOCYTE FROM A POPULATION OF
COMMON ACUTE LYMPHOBLASTIC LEUKEMIA ANTIGEN
(CALLA)-POSITIVE FETAL BONE MARROW CELLS

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T lymphocytes are originally derived from the pluripotential stem cell, as are all hematopoietic cells. The source of precursor T lymphocytes is generally thought to be the bone marrow (apart from the transient hematopoiesis in the fetal liver), from where they migrate to the thymic cortex to initiate T cell differentiation (1). With the advent of the methodology for production of mAbs against T lymphocyte differentiation antigens (2), the unraveling of the T lymphocyte receptor structure (3) and the intrathymic differentiation of T lymphocytes has been elucidated in great detail. In contrast, much less is known about the prethymic T lymphocyte, especially concerning its origin, cell renewal capacity, and surface-marker phenotype. Even though there is ample evidence from T cell malignancies suggesting that the majority of these diseases represent frozen stages of differentiation corresponding to different thymocyte subsets (4, 5), a minority of T cell acute lymphoblastic leukemia cells (T-ALLs) have been found to express the common acute lymphoblastic leukemia (CALLA) antigen (usually defined by mAbs belonging to Cluster of Differentiation (CD) 10 of the Leucocyte Typing Workshops), which is not found in the fetal thymus (5, 6). Even though this antigen is widely distributed in different body tissues, it has been found extremely useful in the differential diagnosis of childhood leukemias, where it has been shown that patients expressing this antigen have a more favorable prognosis within the non-T-ALL subset (7).

We have recently developed a method for purifying a population of immature lymphoid cells from fetal tissues, which express CALLA. Cells obtained by this procedure are morphologically lymphoid and lack mature T cells and myeloid markers. It is believed that these cells represent the normal cellular counterpart of a neoplastic CALLA⁺ pre-B cell seen in non-T-ALL (8). Furthermore, when bulk cultures of such cells are stimulated with PMA or leukocyte-conditioned

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medium, an orderly expression of B cell antigens, including early changes in the surface Ig isotypes, could be demonstrated (9).

Given the fact that not all of these immature lymphoid cells expressed the pan-B antigen B4 (CD 19) (10), which is present on B-lymphoid cells at an even earlier stage than CALLA (9), we hypothesized that within such early lymphoid cells a CALLA⁺ prethymic cell subset could well reside. Since phenotyping experiments had failed to demonstrate known T cell antigens on these cells, we opted to clone them in leucocyte-conditioned medium on selected feeder cells to expand cells with potentials for T cell differentiation. Here we demonstrate that such procedures indeed yielded T cell clones with a mature phenotype. Moreover, we could demonstrate that such cells had passed through a phase of simultaneous expression of T4 or T8 antigens consistent with the already established acquisition of T cell antigens during thymic differentiation.

Materials and Methods

Purification of Fetal Progenitor Cells from Fetal Bone Marrow. Single-cell suspensions from fetal femoral bone marrows were incubated for 30 min at 4°C with a mixture of mAbs reacting with erythroid and myeloid cells as well as mature T lymphocyte antigens: anti-MY7 (CD 13), anti-MY8, anti-MY9, anti-Mol(CD 11), anti-T3(CD 3), anti-T9, and anti-T11(CD 2); for a more thorough description of the reactivities of these antibodies see references 8 and 9. Labeled mononuclear cells were then rosetted with sheep erythrocytes coated with rabbit anti-mouse Ig (kindly donated by J. D. Griffin, Harvard Medical School) by the chromium-chloride method (8). After centrifugation on Ficoll-Hypaque gradients, nonrosetted cells, depleted of erythroid, myeloid cells, as well as mature T cells, were harvested, counted, and analyzed for surface antigens in a standard two-layer immunofluorescence assay. Single-cell suspensions were also prepared from fetal liver and fetal thymus by standard procedures and adult monocytes were prepared from peripheral blood mononuclear cells after plastic adherence for 45 min at 37°C.

Cloning of CALLA⁺ Fetal Progenitor Cells. Nonrosetted cells prepared as described above were labeled with anti-CALLA J5(CD 10) antibodies and FITC-goat anti-mouse antibodies (G/M-FITC). Using the autocloning unit of an Epics V (Coulter Electronics, Hialeah, FL) cell sorter, CALLA⁺ cells were delivered into round-bottomed, 96-well microtiter plates (Costar, Cambridge, MA) containing 2.5×10^4 feeder cells in 200 μ l culture medium (RPMI 1640 supplemented with 10% FCS, Hepes, antibiotics, and 10% leucocyte-conditioned medium prepared as described in reference 11). As often as possible, each combination of CALLA⁺ cells and feeder cells were set up in triplicate in concentrations of CALLA⁺ cells ranging from 1 to 20.

Double-Marker Analysis of Outgrowing Clones. In three separate experiments the microtiter plates containing the CALLA⁺ cells were washed once and all wells were labeled with directly fluoresceinated anti-T4 antibodies (Coulter Immunology), followed by the T8 antibodies labeled with rhodamine, followed by Texas red-avidin. In separate experiments cells were labeled with anti-T6 followed by fluoresceinated goat anti-mouse antibodies and T8-rhodamine followed by Texas red-avidin.

Besides the above phenotyping, which was performed 10 d before macroscopic growth could be observed, phenotyping was also performed after visual growth was observed (usually 17–21 d after setup) on clones that had been expanded separately in 24-well flat-bottomed tissue culture plates (Costar). Such cells were labeled in standard two-layer immunofluorescence assays and analyzed by cell cytometry in a FACS 1 (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The number of cells reactive with a given antibody was calculated after subtracting the reactivity with ascites from a nonproducing hybridoma.

TABLE I
Effect of Different Feeder Cells on Proliferation of Purified Calla⁺
Fetal Bone Marrow Cells

Number of cells per well [‡]	Feeder cells*				
	Autologous			Allogeneic	
	Liver MNC	BM MNC	Thymocytes	Thymocytes	Adult Mo
0	ND, ND [‡]	0, 0	0, 0	0, ND	0, 0
1	0, 0	0, 0	18, 14	9, ND	0, 0
5	0, 0	0, 0	17, 19	13, ND	0, 0
10	0, 0	0, 0	35, 41	20, ND	0, 0

* 2.5×10^4 cells per well; as wells contained 10% LCM in complete culture medium. BM, bone marrow; MNC, mononuclear cells; Mo, monocytes.

[‡] Added by flow cytometry autocloning. Phenotyping of presort BM cells: J5: 87%, 81%; B4: 81%, 73%; T3, T4, T6, T8, and T11: all <0.1%. E_{AET} rosetting: <1:10,000 rosette-forming cells. Phenotypic analysis of sorted cells subsequently allowed to shed CALLA-G/M FITC conjugates at 37°C overnight: J5: >98%, >98%; B4: 88%, 90%; T3, T4, T6, T8, and T11: all <0.1%. E_{AET} rosetting: <1:10,000 rosette-forming cells.

[‡] Number of positive wells in two separate experiments.

TABLE II
Phenotyping of Clones Expanded from Wells Containing
One CALLA⁺ Cell per Well

Exp.	T3/T4/T11 ⁺ , T8 ⁻ *	T3/T8/T11 ⁺ , T4 ⁻ *	T3/T4/T8/T11 ⁺ *
1	3	3	0
2	9	5	0
3	7	6	0
4	4	8	0
5	4	6	0

Delivered using an EPICS-V flow cytometer. 2.5×10^4 irradiated, autologous thymocytes in each well. Precloning phenotypes: J5: 80–92%. T3, T4, T6, T8 and T11: all <0.1%. E_{AET} rosetting: <1:10,000 rosette-forming cells.

* Phenotype of separate analyses of the involved antigens. Positive antigens were present on >90% of the cells from each clone. Negative antigens were absent on >98% of the cells from each clone. All clones were negative for CALLA (J5) and positive for HLA-DR antigens. In no instances were clones with other phenotypes observed.

Results and Discussion

To establish the experimental conditions suitable for expanding cloned CALLA⁺ cells, we delivered one cell to each well in five different 96-well microtiter plates containing different feeder cells using the autocloning accessory of the Epics V cell sorter. As will be seen from Table I, in two separate experiments with five plates each, significant growth could be obtained only from trays containing autologous and (in one experiment only) allogeneic, irradiated (5,600 rad over 25 min) thymocytes.

>90% of such cultures could be expanded into 24-well microtiter plates without feeder cells over 3–5 wk, and when they were subsequently phenotyped, it was apparent that they were all (both autologous, as well as allogeneic thymocytes as feeder cells) of clonal origin representing monomorphic populations of mature T lymphocytes being entirely positive (>95% on all clones) for either the T4(CD4) helper/inducer or the T8(CD8) cytotoxic/suppressor antigen (Table II). Further cell-surface marker analysis performed on these clones showed that they were all positive for HLA-DR antigens and negative for the T6 and CALLA

TABLE III
*Double-Marker Analysis on Day 10 of Viable Cells from 96-Well
 Microtiter Plates with One CALLA⁺ Delivered to Each Well by
 Flow Cytometry Autocloning*

Exp.	T4-FITC/T8- rhodamine-positive Texas red-avidin	T6 + G/M-FITC/T8- rhodamine-positive Texas red-avidin
1	17*	14*
2	22	22
3	27	21

* Percentage double-marker-positive cells after counting at least 50 viable cells in the fluorescence microscope.

antigens, corroborating the mature stage in T cell differentiation of these cells. No clones with other phenotypes (e.g., those of thymocytes, B cells, or myeloid cells) were ever observed.

We believe that these experiments indicate that the cloned CALLA⁺ cells contained a prethymic T lymphocyte lacking known T cell markers. It can, however, be argued that the cloned CALLA⁺ cells might have contained CALLA⁻ immature T cells, but for the following reasons we believe that this is not the case: (a) Reanalysis of CALLA⁺ cells sorted using standard procedures in the FACS V contained >98% CALLA⁺ cells; (b) even before enrichment for CALLA in the Epics V, nonrosetted T3 and T11 depleted cells contained <1% of cells expressing these antigens, as well as T4 and T8; (c) E_{AET} (erythrocytes treated with *S*-(2-aminoethyl)-isothiuronium bromide) rosette formation on nonimmune rosetted cells cultured overnight at 37°C never showed more than 0.1% E_{AET} rosette-forming cells (RFC).

Given these considerations, gross T cell contamination in the sorted suspensions can be safely ruled out, and since the mean percent of wells with growth in five experiments was 11%, these data strongly suggest that the CALLA⁺ cells contained prethymic T cells. To further elucidate the pathway during which these cells had differentiated we performed two-color fluorescence analysis in the fluorescence microscope of all wells of microtiter plates, where one CALLA⁺ cell had been delivered to each well. This was done 10 d after initiation of culture, at a point where no macroscopic growth could be seen, with the object of evaluating the phenotypic markers of cells proliferating at this early time of culture. As will be seen from Table III, cells that expressed the T8 antigen concomitantly with either T4 or T6 antigens could be demonstrated in three separate experiments. In control experiments with wells containing the irradiated thymocytes alone, only nonviable cells with no well-defined immunofluorescence were seen, thus excluding the possibility that the above described fluorescence was due to expansion of irradiation insensitive feeder cells. Thus, we have demonstrated that in cultures containing purified CALLA⁺ cells lacking T cell markers such cells can be induced to differentiate to a stage of dual expression of T4/T8 or T6/T8 to a stage of mature T cell differentiation.

Recently, van Dongen et al. (12) showed the existence of a terminal deoxynucleotidyltransferase-positive (TdT⁺) Tp41⁺ precursor T cell in both adult and childhood bone marrow samples in frequencies of about 5 in 10,000 mononuclear cells. Even though we did not use TdT in this study, it is noteworthy that CALLA⁺ cells purified to homogeneity contained significant amounts (30–50%)

of TdT⁺ cells (8). Furthermore, since the CALLA⁺ constitute 1–3% of fetal mononuclear bone marrow cells, and since 10% of such cells could be cloned, the frequency of our precursor cells is similar to that of van Dongen et al. On the other hand, our approach differs from theirs, since we have chosen to isolate prethymic precursor cells from a pool of cells entirely negative for T cell antigens. Whether the success of cloning T cells from purified CALLA⁺ cells indicates that T cell antigens are acquired in the thymus by T cell antigen–negative cells migrating from the bone marrow cannot be determined, and it is entirely possible that different pathways for prethymic T cells exist involving both the ones described by van Dongen et al. and the one described in this paper.

Summary

We have cloned common acute lymphoblastic leukemia (CALLA)–positive cells from human fetal bone marrow containing <1 in 10,000 E-RFC in round-bottomed microtiter wells (one cell per well) using the autocloning unit of an EPICS-V cell sorter. Expansion of such cells (with IL-2 and heavily irradiated autologous thymocytes as feeder cells) resulted in growth in 6–14% of the wells (mean, 11%) with cells with mature T lymphocyte phenotype. Two-color fluorescence analysis of outgrowing cultures furthermore ascertained that these cells had differentiated through a phase of simultaneous expression of T4 and T8 antigens and at the same time expression of the thymocyte-associated T6 antigens. Thus, given the fact that 10–20% of T cell acute lymphoblastic leukemia (T-ALLs) are CALLA⁺, we have been able to identify a human prethymic T lymphocyte population that might be the normal counterpart of precursor cell to the CALLA⁺ T-ALL cell.

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References

1. Reinherz, E. L., P. C. Kung, G. Goldstein, R. H. Levey, and S. F. Schlossman. 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc. Natl. Acad. Sci. USA.* 77:1588.
2. Reinherz, E. L., and S. F. Schlossman. 1980. The differentiation and function of human T lymphocytes. *Cell.* 19:821.
3. Meuer, S. C., O. Acuto, R. E. Hussey, J. C. Hodgdon, K. A. Fitzgerald, S. F. Schlossman, and E. L. Reinherz. 1983. Evidence for the T3-associated 90K heterodimer as the T-cell antigen receptor. *Nature (Lond.).* 303:808.
4. Umiel, T., J. F. Daley, A. K. Bhan, R. H. Levey, S. F. Schlossman, and E. L. Reinherz. 1982. Acquisition of immune competence by a subset of human cortical thymocytes expressing mature T cell antigens. *J. Immunol.* 129:1054.
5. Rosenthal, P., I. J. Rimm, T. Umiel, J. D. Griffin, R. Osathanodh, S. F. Schlossman, and L. M. Nadler. 1983. Ontogeny of human hematopoietic cells: analysis utilizing monoclonal antibodies. *J. Immunol.* 131:232.
6. Foon, K. A., and R. F. Todd, III. 1986. Immunologic classification of leukemia and lymphoma. *Blood.* 68:1.

7. Chessels, J. M., R. M. Hardisty, N. T. Rapson, and M. F. Greaves. 1977. Acute lymphoblastic leukaemia in children: Classification and prognosis. *Lancet*. ii:1307.
8. Hokland, P., P. Rosenthal, J. D. Griffin, L. M. Nadler, J. F. Daley, M. Hokland, S. F. Schlossman, and J. Ritz. 1983. Purification of fetal hematopoietic cells which express the common acute lymphoblastic leukemia antigen (CALLA). *J. Exp. Med.* 157:114.
9. Hokland, P., J. Ritz, S. F. Schlossman, and L. M. Nadler. 1985. Orderly expression of B cell antigens during the *in vitro* differentiation of nonmalignant human pre-B cells. *J. Immunol.* 135:1746.
10. Nadler, L. M., J. Ritz, R. Hardy, J. M. Pesando, and S. F. Schlossman. 1981. B4, a human B lymphocyte associated antigen expressed on normal, mitogen activated, and malignant B lymphocytes. *J. Immunol.* 131:244.
11. Todd, III, R. F., J. D. Griffin, J. Ritz, L. M. Nadler, T. Abrams, and S. F. Schlossman. 1981. Expression of natural monocyte macrophage differentiation antigens on HL-60 promyelocytes undergoing differentiation induced by leukocyte conditioned medium or phorbol diester. *Leuk. Res.* 5:491.
12. van Dongen, J. J. M., H. Hooijkaas, M. Comans-Bitter, K. Hählen, A. de Klein, G. E. van Zanen, M. B. van't Veer, J. Abels, and R. Benner. 1985. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. *J. Immunol.* 135:3144.