# PURIFICATION AND CHARACTERIZATION OF FETAL HEMATOPOIETIC CELLS THAT EXPRESS THE COMMON ACUTE LYMPHOBLASTIC LEUKEMIA ANTIGEN (CALLA)*

By PETER HOKLAND,‡ PAUL ROSENTHAL, JAMES D. GRIFFIN,§ LEE M. NADLER,‖ JOHN DALEY, MARIANNE HOKLAND,¶ STUART F. SCHLOSSMAN, and JEROME RITZ**

*From the Division of Tumor Immunology, Sidney Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115*

Previous studies using conventional heteroantisera and monoclonal antibodies (1, 2) have characterized the expression of a common acute lymphoblastic leukemia antigen (CALLA)[1] in various hematopoietic malignancies and normal tissues. CALLA has been demonstrated to be a 100,000-dalton glycoprotein that is expressed by leukemic cells from ~80% of patients with non-T acute lymphoblastic leukemia (ALL), 10% of patients with T-ALL, and 40% of patients with chronic myelocytic leukemia (CML) in blast crisis, as well as lymphoma cells from ~40% of patients with T cell lymphoblastic lymphomas and almost all Burkitt's lymphomas and nodular lymphomas (3). Within the hematopoietic system, CALLA is also detected on a small population of mononuclear cells (1–3%) in either normal or regenerating bone marrow. Fetal hematopoietic organs contain a larger proportion of CALLA+ cells and mononuclear cell suspensions from fetal liver and fetal bone marrow have been found to contain 2–10% CALLA+ cells (2–5).

Although much is known about the distribution of CALLA, very little is known about its functional role on the cell surface or its appearance during ontogeny. Recent studies have demonstrated that CALLA belongs to a family of 100,000-dalton cell surface proteins that are widely expressed by hematopoietic cells (6) and is also present in some nonhematopoietic tissues (7). In addition, binding of J5 monoclonal antibody to cell surface antigen results in the modulation of the expression of CALLA on

* Supported in part by grant CA-28704 from the National Institutes of Health.

‡ On leave from the University Department of Medicine and Haematology, University of Aarhus, Denmark. Research Fellow of the Danish Medical Research Council.

§ Research Fellow of the Medical Foundation, Boston, MA and the Joanna C. Wood Foundation, Philadelphia, PA.

‖ Research Fellow of the Medical Foundation, Boston, MA.

¶ Research Fellow of the Danish Cancer Society.

** Special Fellow of the Leukemia Society of America.

[1] *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; BM, bone marrow; C, complement; CALLA, common acute lymphoblastic leukemia antigen; CFU-G/M, granulocyte/monocyte colony-forming unit; CML, chronic myelocytic leukemia; cyto-$\mu$, intracytoplasmic $\mu$ chain; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; G/M-FITC, fluorescein-conjugated goat anti-mouse immunoglobulin; G/M-TRITC, rhodamine-conjugated goat anti-mouse immunoglobulin; HBSS, Hanks' balanced salts solution; MoAb, monoclonal antibodies; R/M-SRBC, rabbit anti-mouse Ig-coated sheep erythrocytes; SMEM + 2.5% AB, supplemented minimum essential medium with 2.5% human AB; SRBC, sheep erythrocytes; TdT, terminal transferase enzyme.



J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/01/0114/16 $1.00
Volume 157 January 1983 114–129

leukemic cells and cell lines. J5 modulation occurs in vitro (8) and in vivo (9) and results in a reversible and specific loss and internalization of cell surface CALLA (10). Despite the analogous modulatory behavior of CALLA and known membrane receptors for various hormones, no functional role for CALLA has yet been defined.

Given the compelling data demonstrating that most tumors of myeloid and lymphoid systems reflect phenotypes of frozen stages of differentiation corresponding to their normal cellular counterparts, in this study we have purified normal fetal hematopoietic cells that express CALLA to further delineate the role of this cell surface glycoprotein in early lymphoid differentiation. Pure populations of CALLA+ cells were obtained by either immune rosetting or fluorescence-activated cell sorting, and these cells were subsequently characterized using dual fluorescence labeling techniques for the presence of specific cytoplasmic and surface markers. These data suggest that CALLA+ cells are committed lymphoid cells that are in the early stages of lymphoid differentiation and that, furthermore, normal CALLA+ cells appear to be the normal counterparts of CALLA+ lymphoblastic leukemia cells.

## Materials and Methods

*Preparation of Mononuclear Single Cell Suspensions from Fetal Tissues.* Fetal tissue specimens were obtained immediately after prostaglandin- or saline- induced abortions from fetuses with no apparent abnormalities. The age of the fetus was determined by measurements of crown-rump and foot lengths. Procurance of all specimens was approved by the Brigham and Women's Hospital Committee on the Use of Human Subjects in Research, Boston, MA.

Fetal bone marrow was obtained from femoral bones that were removed aseptically. Single cell suspensions were produced by flushing the intramedullary cavities with Hanks' balanced saline solution (HBSS). Fetal livers were minced into 1-$cm^3$ pieces and passed repeatedly in and out of a 20-$cm^3$ syringe until large clumps of cells were no longer visible. Subsequently, cells were passed through a cone-shaped nylon mesh to obtain single cell suspensions.

Single cell suspensions from both organs were then layered onto Ficoll-Hypaque (F/H) density gradients and after centrifugation the resulting interface cells were washed twice and resuspended in supplemented minimum essential medium with 2.5% pooled human AB serum (SMEM + 2.5% AB). For preparation of fetal liver mononuclear cell suspensions, this step was repeated to maximize elimination of erythrocytes.

*Monoclonal Antibodies.* The production and reactivities of the monoclonal antibodies (MoAb) used in this study have been described previously. A summary of the specificity of these reagents is presented in Table I.

*Preparation of Rabbit Anti-Mouse Ig-coated Sheep Erythrocytes (R/M-SRBC).* As described previously (23), New Zealand white rabbits were immunized repeatedly with a mixture of MoAb including J2, J5, J13, MY7, and MY8. The immune serum was first passed through a column of human Ig coupled to Sepharose 4B (Pharmacia) and then through a column of mouse Ig-Sepharose. Purified rabbit anti-mouse Ig was eluted from the second column with 1 M glycine buffer, pH 2.0, extensively dialyzed against 0.9% saline solution, adjusted to a concentration of 1 mg protein/ml, and stored at −20°C.

Sheep erythrocytes (SRBC), washed three times in saline, were coupled with the rabbit anti-mouse antiserum with $CrCl_3$ as follows: 0.5 ml packed SRBC were mixed with 0.5 ml rabbit anti-mouse antiserum and 0.5 ml saline, whereupon 0.5 ml of freshly prepared $CrCl_3$ solution (1 mg/ml) was added dropwise under vigorous stirring. After a 6–7-min incubation at room temperature, the reaction was stopped with cold phosphate-buffered saline and the coupled SRBC were washed three times in saline and resuspended to a 10% solution in SMEM + 2.5% AB.

*Immune Rosette Depletion of Myeloid and Erythroid Cells.* To deplete the mononuclear cell suspensions of erythroid and myeloid precursor cells, mononuclear cells from fetal bone marrow

TABLE I

*Summary of Monoclonal Antibodies Used in the Purification and Characterization of Fetal Hematopoietic Cells*

| Monoclonal antibody | Molecular weight* | Hematopoietic cellular reactivity | Reference |
|---|---|---|---|
| J5/J13 | 100,000 | Early lymphoid cells, common ALL cells, blast-crisis lymphoid CML, Burkitt's lymphoma, nodular lymphoma | 2, 3[2] |
| J2 | 26,000 | Activated T cells, some thymocytes, platelets, early lymphoid cells, ALL cells | 11 |
| I-2 (Ia antigen) | 29,000<br>34,000 | B cells, monocytes, activated T cells, early hematopoietic cells | 12 |
| B1 | 30,000 | All B cells except for plasma cells, B cell tumors except for myeloma, some non-T ALL cells | 13, 14 |
| Mo1 | 94,000<br>155,000 | Monocytes, null cells, granulocytes, acute monocytic leukemia | 15, 16 |
| Mo2 | 55,000 | Monocytes, acute monocytic leukemia | 15, 16 |
| MY7 | 160,000 | Differentiated granulocytic and monocytic cells, acute myelocytic leukemia cells | 17 |
| MY8 | — | | |
| T3 | 20,000 | Mature T cells | 18, 19 |
| T9 (transferrin receptor) | 94,000 | Activated T cells, rapidly proliferating cells, early erythroid cells | 19, 20, 21 |
| T10 | 45,000 | Activated T cells, rapidly proliferating cells, early lymphoid cells, plasma cells | 19, 21 |
| T11 (E rosette receptor) | 55,000 | T cells, thymocytes | 22 |

* Apparent molecular weight of antigen under reducing conditions in sodium dodecyl sulfide-polyacrylamide gel electrophoresis.

and fetal liver were incubated at 4°C for 30 min with a mixture of MoAb including: T9 (dilution 1:2,000), Mo1 (1:100), Mo2 (1:100), MY7 (1:100), and MY8 (1:100). After three washes in SMEM + 2.5% AB, the cells were resuspended to 1.0 ml and mixed with 1.0 ml of the 10% R/M-SRBC solution. When leukocyte cell numbers exceeded $200 \times 10^6$, 1–3 ml of R/M-SRBC was used. Using this method, up to $600 \times 10^6$ cells can be processed in one tube. After a centrifugation at 180 *g* for 5 min, the rosette mixture was resuspended with a Pasteur pipette until clumps were no longer visible. The quality of the rosettes was determined under fluorescence microscopy in an acridine orange-stained aliquot; subsequently, the rosettes were layered on F/H and spun for 15 min at 1,140 *g*. The resulting interface suspension (subsequently referred to as MoAb-rosette-negative cells) was harvested and washed, whereas the pellet (MoAb-rosette-positive cells) was recovered after lysis of SRBC with ammonium chloride buffer. The viability in each fraction was >90% and total recovery of cells was >60%.

*Purification of CALLA+ Cells by Rosette Sedimentation.* CALLA+ cells were purified by a second rosette sedimentation after labeling of the MoAb-rosette-negative cell fractions with anti-CALLA antibodies. Pilot experiments with CALLA+ ALL lines (Nalm-1 and Laz 221) had shown that the rosetting efficiency could be increased by adding an IgM antibody, J13, which is also specific for CALLA, to J5 during the antibody-labeling procedure. Consequently, an equal mixture of J5 and J13 (final dilution of each, 1:100) was used when the MoAb-rosette-negative cell fraction was to be rosetted. Otherwise, the rosetting was performed as described above.

*Purification of CALLA+ Cells by Fluorescence-activated Cell Sorting.* In some experiments, CALLA+ cells were purified from MoAb-rosette-negative fractions by fluorescence-activated

[2] Greaves, M. F., G. Hariri, R. A. Newman, D. R. Sutherland, M. A. Ritter, and J. Ritz. Selective expression of the common acute lymphoblastic leukemia (gp100) antigen on immature lymphoid cells and their malignant counterparts. *Blood.* In press..

cell sorting after labeling with J5 antibody and a 1:50 dilution of fluorescein-conjugated goat anti-mouse antiserum (G/M-FITC; Tago Inc., Burlingame, CA) for 30 min at 4 C. After two washes the cells were separated into J5+ and J5− (CALLA+ and CALLA−) subpopulations using an EPICS V (Coulter Electronics Inc., Hialeah, FL). Post-sort viability in both fractions was >95%, and the purity of the subsets was >97% by reanalysis on the EPICS V.

*Phenotypic Analysis of CALLA+ and CALLA− Cell Fractions.* Dual fluorescence techniques were used to obtain direct evidence for the coexpression of other antigens on the CALLA+ cells. With sorted CALLA+ cells that were labeled with J5 and G/M-FITC, capping of CALLA-J5-G/M-FITC complexes was observed after an overnight incubation at 37°C. CALLA+ cells purified by J5 rosette sedimentation were labeled with G/M-FITC before overnight incubation to induce capping of J5-CALLA complexes.

After overnight incubation, the CALLA+ cells were incubated with appropriate dilutions of a second monoclonal antibody followed by addition of rhodamine-conjugated goat anti-mouse antisera (G/M-TRITC, 1:40 dilution) (Sigma Chemical Co., St. Louis, MO). For detection of the B1 antigen, biotin-labeled anti-B1 (1:20) and rhodamine-conjugated avidin (1:80; Sigma Chemical Co., St. Louis, MO) was used. A minimum of 200 cells were counted in a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York) equipped with filter combinations that allowed for the clear distinction between fluorescein caps (J5-CALLA) and rhodamine membrane staining (second antibodies).

The CALLA-negative cells, which contained no interfering antibodies, were analyzed for reactivity with specific monoclonal antibodies by indirect immunofluorescence. Cells were incubated with monoclonal antibody at 4°C for 30 min, washed twice, and then incubated with G/M-FITC for 30 min at 4°C. 10,000 cells were then analyzed for each reagent on a fluorescence-activated cell sorter (FACS-I; B-D FACS Systems, Mountain View, CA). Background fluorescence was determined by incubating identical cells with ascites fluid from a nonreactive hybridoma (J0).

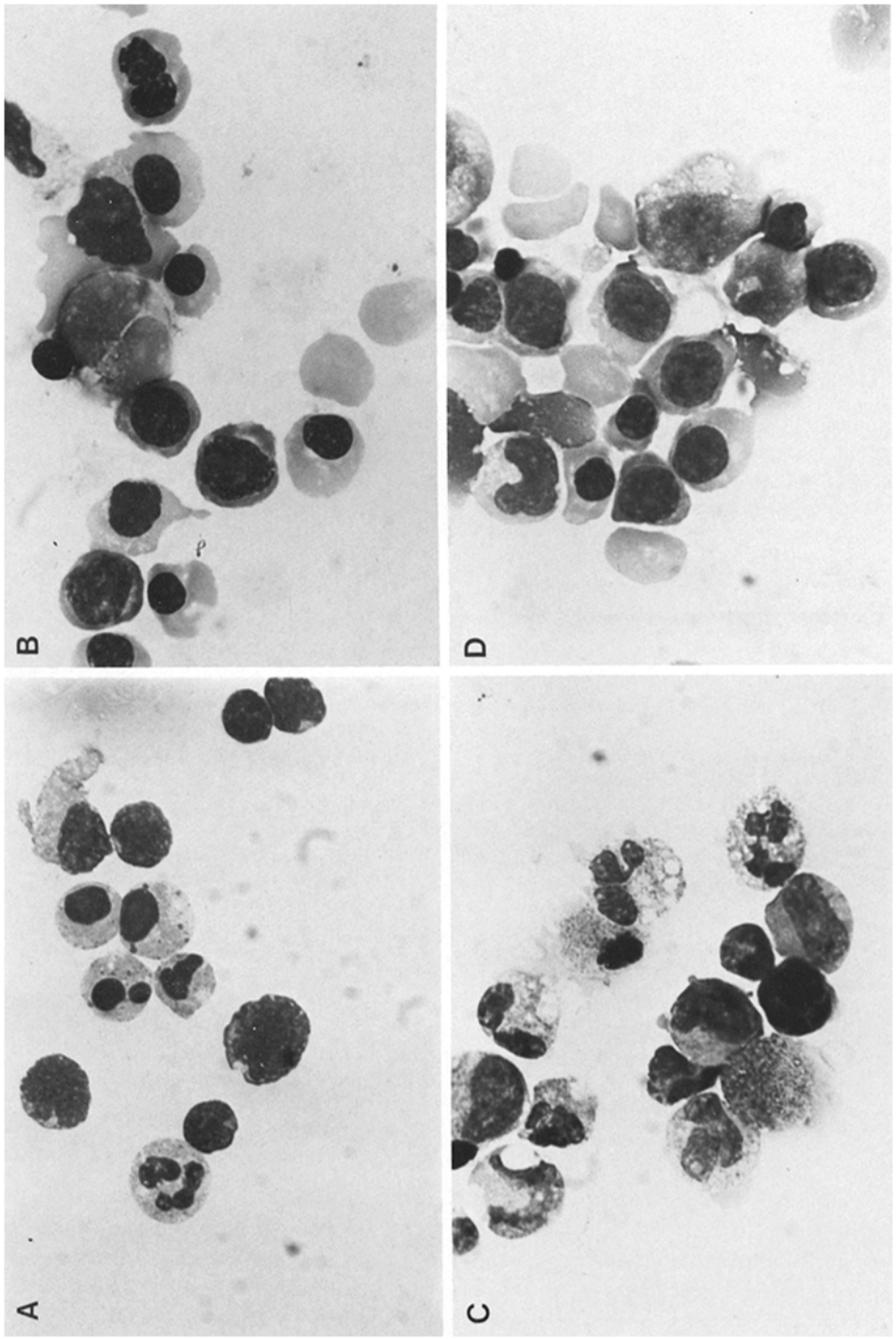
*Detection of Intracellular Antigens.* The terminal transferase enzyme (TdT) was detected by a two-layer immunofluorescence assay on cytocentrifuged, methanol-fixed cells as described by Bollum (24) using a kit from Bethesda Research Laboratories (Rockville, MD). Intracytoplasmic $\mu$ (cyto-$\mu$) was detected on cytocentrifuged, methanol-fixed smears using directly fluorescein- or rhodamine-conjugated rabbit anti-$\mu$ chain antisera (kindly provided by Dr. M. D. Cooper, University of Alabama, Birmingham, AL). Nalm-1 cells were used as positive controls for the intracellular markers in the TdT assay and Raji cells in the cyto-$\mu$ assay.

*Modulation In Vitro of the CALLA+ Cells.* Antigenic modulation in vitro was induced in MoAb-rosette-negative cell fractions by addition of monoclonal antibody and subsequent incubation for different periods of time at 37°C. Either J5 antibody, J0, or anti-Ia was added to identical cultures. To ensure antibody excess, 0.1 mg antibody per $10^6$ cells was used. Cell culture media consisted of RPMI 1640 supplemented with 10% fetal calf serum (FCS). After incubation, cells were washed twice and phenotyped as described above for the CALLA− cells.

*Myeloid Precursor Cell (CFU-G/M) Assays.* CFU-G/M were assayed as described by Griffin et al. (23) by plating $10^5$ mononuclear cells/ml in Iscove's modified Dulbecco's minimal essential medium containing 20% FCS and 0.3% agar (Agar Noble; Difco, Detroit, MI), over a feeder layer of $1 \times 10^6$ peripheral blood leukocytes/ml in 0.5% agar. After 10 d of culture at 37°C in an atmosphere of 5% $CO_2$ in humidified air, colonies (>40 cells) were enumerated using an inverted microscope.

## Results

*Purification of CALLA+ Cells from Fetal Tissues.* As shown in Fig. 1 A, mononuclear cells obtained from fetal bone marrow after F/H density gradient sedimentation primarily consisted of maturing myeloid cells. In contrast, mononuclear cells obtained from fetal liver (Fig. 1 E) after two F/H-purification steps consisted primarily of maturing erythroid precursors. However, in both cases these cells could be removed from the mononuclear cell suspensions by rosette sedimentation with a mixture of MoAb (Fig. 1 B and F). In contrast to the MoAb-rosette-positive fractions, the MoAb-


B
D
A
C

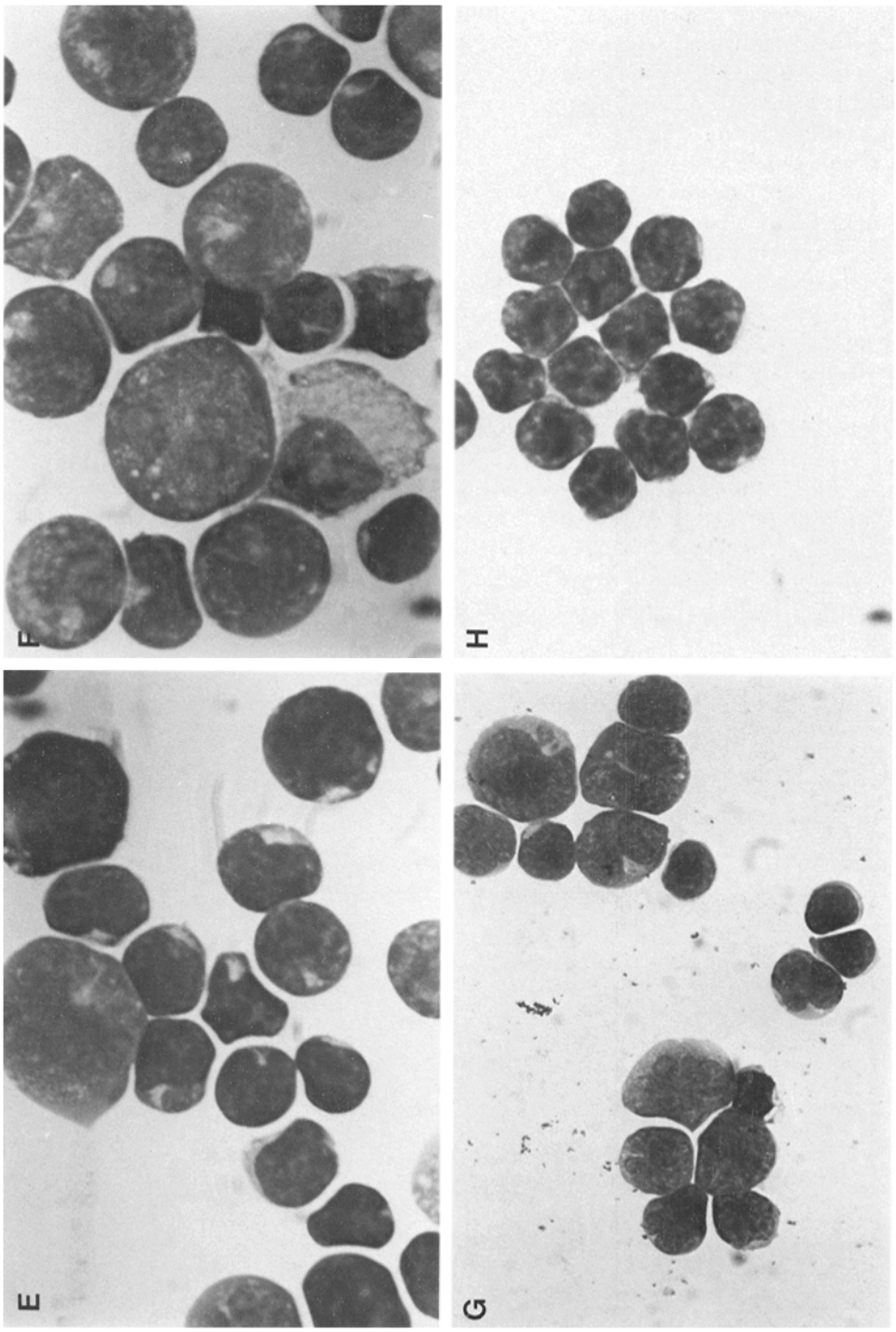



FIG. 1. Photomicrographs of Giemsa-stained smears from different fetal cell fractions. A, F/H-purified BM cells; × 650. B, F/H-purified liver cells; × 650. C, MoAb-rosette-positive BM cells; × 650. D, MoAb-rosette-positive liver cells; × 650. E, MoAb-rosette-negative BM cells; × 1,000. F, MoAb-rosette-negative liver cells; × 1,000. G, MoAb-rosette-negative, CALLA− cells; × 650. H, FACS-purified CALLA+ cells; × 1,000.

rosette-negative suspensions from both bone marrow (Fig. 1 C) and liver (Fig. 1 G) were remarkably similar, consisting of a mixture of small to medium-sized lymphocytes and large blast cells. When separating the MoAb-rosette-negative fraction further into CALLA+ and CALLA− subsets, the large blast cells were found exclusively in the CALLA− fraction (Fig. 1 D), whereas the CALLA+ fraction consisted solely of lymphoid-appearing cells (Fig. 1 H).

After rosette depletion of mature myeloid cells, 40–90% of the cells in MoAb-rosette-negative bone marrow suspensions were CALLA+. Further purification of CALLA+ cells was then accomplished by fluorescence-activated cell sorting using an EPICS V. A representative purification experiment is shown in Fig. 2 where the percentage of CALLA+ cells in the MoAb-rosette-negative subset was 85%; subsequent purification yielded a population that was 98% CALLA+.

To purify CALLA+ cells from fetal liver, an alternative method that was able to process larger numbers of cells was used. After two F/H sedimentations and rosette depletion of mature cells with a mixture of MoAb, the MoAb-rosette-negative fetal liver suspensions contained 20–60% CALLA+ cells. Subsequently, CALLA+ cells were purified from the MoAb-rosette-negative fraction by indirect immune rosetting with both J5 and J13 antibodies. Table II presents the results of a representative experiment in which the CALLA+ fetal liver cells from a 22-wk-old fetus were purified by two consecutive rosette sedimentations. In the depletion step with MoAb-rosettes directed against a mixture of erythroid and myeloid differentiation antigens, >90% of the cells were removed. Of the remaining cells, approximately one-half were CALLA+, and after labeling these MoAb-rosette-negative cells with J5 and J13 antibodies, they could be separated into CALLA+ and CALLA− fractions by a second rosetting with R/M-SRBC. The yields in each of the rosette sedimentations ranged from 45 to 70%.

*Surface Antigens Expressed by CALLA+ and CALLA− Cells.* The persistance of J5-G/M-FITC complexes as caps on sorted and rosetted CALLA+ cells (after addition of

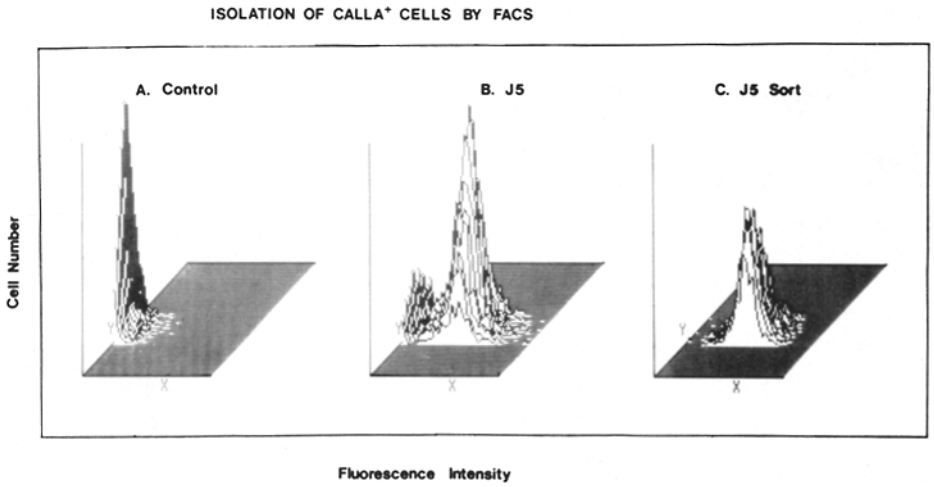



FIG. 2. Isolation of CALLA+ cells by cell sorting on an EPICS V cell sorter. X-axis, relative fluorescence intensity. Y-axis, cell number (10,000 cells analyzed per panel). Z-axis, light scatter (relative cell size). A, MoAb-rosette negative BM cells labeled with G/M-FITC. B, same cells as in A labeled with J5. C, reanalysis of sorted J5+ cells.

TABLE II

*Purification of CALLA+ Cells from Fetal Liver by Immune Rosette Sedimentations*

| Cell fraction | Total cells | Total J5+ cells | Percent J5+ cells* |
|---|---|---|---|
| | × $10^6$ | × $10^6$ | |
| Unseparated‡ | 510 | 35.7 | 7 |
| MoAb-rosette positive§ | 320 | 6.4 | 2 |
| MoAb-rosette negative§ | 38 | 20.1 | 53 |
| MoAb-rosette negative, J5−‖ | 12 | 0.4 | 3 |
| MoAb-rosette negative, J5+‖ | 11 | 10.5 | >95 |

* Determined by indirect immunofluorescence assay and FACS analysis.

‡ Mononuclear cell suspensions purified two times by F/H density-gradient sedimentation.

§ Cells obtained from interfaces (rosette negative) or pellets (rosette positive) after R/M-SRBC immune rosette sedimentation with cells labeled simultaneously with Mo1, Mo2, MY7, MY8, and T9 monoclonal antibodies.

‖ Rosette-negative cell suspensions obtained after depletion with Mo1, Mo2, MY7, MY8, and T9 monoclonal antibodies and R/M-SRBC were incubated with J5 antibody and R/M-SRBC. J5− (interface cells) and J5+ (pellet cells) populations were then separated by an additional immune rosette sedimentation.

TABLE III

*Phenotypic Analysis of CALLA+ Cells from Fetal Hematopoietic Tissues with Monoclonal Antibodies*

| Cell source | Age | Percent positive cells* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | J2 | J5 | J13 | Ia | T10 | B1‡ | MY7/ MY8§ | T9 | T3/ T11§ |
| | *wk* | | | | | | | | | |
| Bone marrow | 22‖ | >95 | >95 | NT** | >95 | >95 | 45 | NT | NT | NT |
| | 22‖ | >95 | >95 | >95 | >95 | >95 | 41 | <5 | NT | NT |
| | 23‖ | NT | >95 | NT | >95 | >95 | 52 | NT | NT | NT |
| Liver | 21¶ | >95 | >90 | >95 | >95 | >95 | 31 | <5 | <5 | <5 |
| | 22‖ | >95 | >95 | >95 | >95 | >95 | 39 | <5 | <5 | <5 |
| | 23¶ | >95 | >95 | >95 | >95 | >95 | 30 | <5 | <5 | <5 |
| | 24¶ | NT | >90 | NT | >95 | >95 | 27 | NT | NT | NT |

* Determined by fluorescence microscopy of J5+ cells incubated overnight at 37°C with G/M-FITC to obtain capping and subsequently stained with a second monoclonal antibody and G/M-TRITC.

‡ Labeled with biotin-conjugated B1 followed by rhodamine-labeled avidin.

§ Labeled simultaneously with both monoclonal antibodies noted.

‖ Purified from MoAb-rosette-negative cell fractions by cell sorting.

¶ Purified from MoAb-rosette-negative cell fractions by J5 rosette sedimentation.

** Not tested.

G/M-FITC to the latter) allowed for the definition of other cell surface antigens on these cells by a second monoclonal antibody and G/M-TRITC. The results from a series of experiments with both fetal bone marrow and fetal liver of different ages are given in Table III. Although these cells have been isolated from different tissues by two different methods, the cells displayed similar cell surface antigens. The purity of each suspension, as judged by the presence of J5-FITC caps, exceeded 90% in every instance. It should also be noted that addition of more J5 antibody and G/M-TRITC

did not give any additional fluorescence, indicating that all of the J5 surface antigen was located within the G/M-FITC cap and that these cells had not synthesized more CALLA during the overnight culture. Further analysis with J13 antibody, which is also specific for CALLA, showed that nearly all the cells bound J13 within the J5-FITC caps. There was also no appreciable cell surface staining with J13 antibody other than that found in the J5 caps.

Three antigens, Ia, J2, and T10, were found on virtually all the CALLA+ cells. In contrast to the reactivity pattern seen with J13, these antigens were uniformly distributed along the cell membrane and were distinct from CALLA. The B1 antigen, in contrast, was only found on a fraction of the CALLA+ cells, and, when present, was uniformly distributed on the cell surface. Very few CALLA+ cells were reactive with anti-myeloid or T cell antibodies. Thus, virtually all CALLA+ fetal bone marrow and liver cells were J2+, Ia+, T10+, MY7/8−, T3/9/11−, whereas 30–50% expressed B1.

The phenotype of the CALLA− cells obtained after J5/J13 rosette sedimentation of MoAb-rosette-negative fractions was determined by indirect immunofluorescence with subsequent analysis on a FACS-I. As shown in Table IV, these cells have been effectively depleted of CALLA+ cells, as judged by the low numbers of cells reactive with the J5 and J13 antibodies. Likewise, very few myeloid or mature T cells were found. On the other hand, significant percentages of these cells expressed T10. In contrast to the analysis of CALLA+ cells, only a minority of the CALLA− cells were reactive with either J2 or anti-B1.

The CALLA+ and CALLA− populations were also analyzed for the presence of nuclear TdT and cyto-$\mu$ after methanol fixation. As shown in Table V, 10–30% of the CALLA+ cells express cyto-$\mu$ chains, but this marker is not restricted to the CALLA+ subset because 10–15% of CALLA− cells also contain cyto-$\mu$. Furthermore, the finding of cyto-$\mu$+ cells within the CALLA− subset correlates rather closely with the demonstration of 11–17% B1+ cells within this population (Table IV). When expression of TdT was examined, it was found that 5–25% of CALLA+ cells exhibited a nuclear TdT staining pattern. In the CALLA− population, a somewhat higher percentage of cells (35–50%) was TdT positive. In further experiments, biotin-labeled B1 and rhodamine-avidin were combined with the intracellular markers in CALLA+ cells. As summarized in Table VI, it was determined that nearly all of the B1+ cells were

TABLE IV

*Phenotypic Analysis of MoAb-Rosette-negative, CALLA− Cells Isolated from Fetal Liver*

| Age of fetus | Percent positive cells* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | J2 | J5 | J13 | Ia | T10 | B1 | MY7/MY8‡ | T9 | T3/T11‡ |
| *wk* | | | | | | | | | |
| 22 | 23 | 3 | 5 | 56 | 74 | 13 | <1 | 0 | 2 |
| 22 | 16 | <1 | 6 | 47 | 57 | 11 | <1 | 4 | <1 |
| 23 | 33 | 2 | 2 | 63 | 49 | 17 | 6 | NT§ | 6 |
| 27 | 13 | 3 | 2 | NT | 54 | 16 | 5 | NT | NT |

* Determined by indirect immunofluorescence assay and FACS analysis.
‡ Labeled simultaneously with both monoclonal antibodies noted.
§ Not tested.

TABLE V

*Intracellular Markers of Purified CALLA+ and CALLA− Fetal Cells*

| Cell source | Age | CALLA | Cyto-μ* | TdT* |
|---|---|---|---|---|
| | *wk* | | | |
| Bone marrow | 22 | +‡ | 17 | 7 |
| Bone marrow | 23 | + | 23 | 16 |
| Liver | 21 | + | 14 | 7 |
| Liver | 22 | + | 27 | 19 |
| Liver | 23 | + | 21 | 21 |
| Liver | 22 | −§ | 12 | 43 |
| Liver | 23 | − | 10 | 39 |
| Liver | 27 | − | 15 | 47 |

* Percent positive cells determined by immunofluorescence microscopy. 200 cells were scored in each experiment.
‡ +, MoAb-rosette negative, CALLA+.
§ −, MoAb-rosette negative, CALLA−.

TABLE VI

*Double Marker Analysis of CALLA+ Cells with B1 Antibody and Intracellular Antigens*

| Subset | Tissue | Age | TdT+ | TdT− | Cyto-μ+ | Cyto-μ− |
|---|---|---|---|---|---|---|
| | | *wk* | | | | |
| B1 positive | Bone marrow | 23 | 6 | 94* | 44 | 56 |
| | Liver | 23 | 13 | 87 | 28 | 72 |
| B1 negative | Bone marrow | 23 | 28 | 72 | 2 | 98 |
| | Liver | 23 | 32 | 68 | 1 | 99 |

* At least 200 cells were scored by fluorescence microscopy and the percentage of cells positive and negative for a specific marker was subsequently calculated.

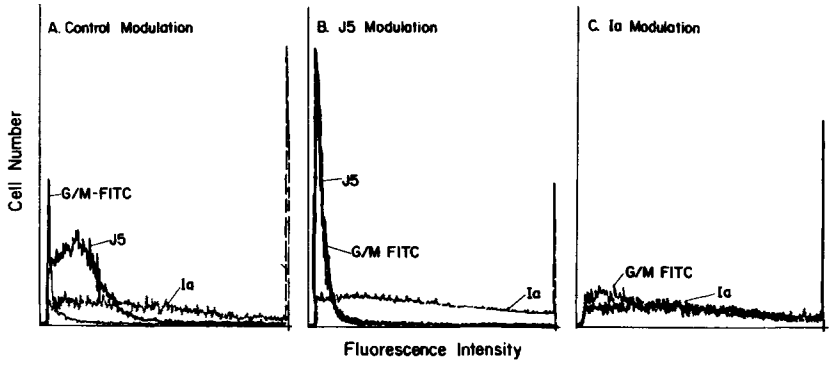



FIG. 3. Modulation of CALLA in vitro. MoAb-rosette-negative BM suspensions were incubated at 37°C for 24 h with ascites from the nonreactive clone J0 (A), with J5 (B), or with the anti-Ia antibody I2 (C). Subsequently, cells were labeled with either J5 or I2, followed by G/M-FITC, or G/M-FITC alone (A) as control.

TABLE VII

*Effect of CALLA Modulation by J5 Antibody In Vitro on Expression of Unrelated Surface Antigens*

| Incubation with J5 | Percentage positive cells* | | | | | |
|---|---|---|---|---|---|---|
| | J2 | J5 | J13 | T10 | B1 | Ia |
| *h* | | | | | | |
| 0 | NT‡ | 64 | NT | NT | 19 | 82 |
| 24 | 87 | 6 | 4 | 91 | 21 | 92 |
| 48 | 81 | 2 | 4 | 95 | 19 | 96 |

* Determined by indirect immunofluorescence assay and FACS analysis.
‡ Not tested.

TABLE VIII

*Analysis of CFU-G/M Cells*

| Specimen | Subset* | Colonies/$10^5$ cells‡ | Percent yield§ |
|---|---|---|---|
| Liver | Unseparated cells | 980 | — |
| | MoAb-rosette negative, CALLA− | 1,600 | 56.0 |
| | MoAb-rosette negative, CALLA+ | 50 | 0.4 |
| Bone marrow | Unseparated cells | 220 | — |
| | MoAb-rosette positive | 70 | 18.5 |
| | MoAb-rosette negative | 720 | 78.5 |
| | MoAb-rosette negative, CALLA− | 2,100 | 76.3 |
| | MoAb-rosette negative, CALLA+ | 50 | 0.1 |
| Liver | Unseparated cells | 650 | — |
| | MoAb-rosette positive | 53 | 5.8 |
| | MoAb-rosette negative | 1,600 | 45.7 |
| | MoAb-rosette negative, CALLA+ | 16 | 0.0 |

* For explanation, see legend to Table II.
‡ Scored day 10.
§ Based on the number of colonies compared with unseparated cells.

found in the TdT− subset and that the cyto-$\mu$+ cells were a subset of the B1+ cells. This suggests that there is very little overlap between subsets expressing either TdT or cyto-$\mu$ within CALLA+ fetal cells.

*Modulation of CALLA Expression.* Previous studies using leukemic cells and cell lines have demonstrated that binding of J5 monoclonal antibody to cell surface CALLA results in the specific modulation of this cell surface antigen. We were therefore interested in evaluating the relevance of this phenomenon using nonmalignant CALLA+ cells. MoAb-rosette-negative bone marrow (BM) suspensions highly enriched for CALLA+ cells were incubated for various periods of time with either J0 (ascites from a nonreactive hybridoma), J5, or anti-Ia, which has previously been shown not to modulate Ia antigen expression (8). As can be seen in Fig. 3A, 65% of the unmodulated cells were CALLA+ and 85% bore Ia antigens. After incubation with J5 at 37°C, 80% of the CALLA+ cells no longer expressed either surface antigen or J5 antibody. However, these cells continued to express Ia antigen. In contrast, the Ia antibodies did not affect the expression of Ia antigen (Fig. 3C). Using a larger

panel of MoAb to analyze the J5-modulated cells (Table VII), it was seen that the decrease in J5 reactivity was accompanied by a similar decrease in J13 expression. In contrast, only minor changes were seen in the expression of the non-CALLA-related antigens B1, J2, T10, and Ia even when the incubation period was extended to 48 h.

*CFU-G/M Assays.* Previous studies using adult bone marrow or peripheral blood depleted of CALLA+ cells by J5 and complement (C) treatment have shown that such procedures did not selectively deplete this population of committed myeloid precursor cells (CFU-C, BFU-E, CFU-E, CFU-G/E) (25). In Table VIII, data from experiments where different purified fractions of fetal liver and fetal bone marrow were tested for activity in CFU-G/M assays are shown. Irrespective of the tissue source and the method of purification, the CALLA+ cell fractions contained virtually no CFU-G/M. In contrast, most of the CFU-G/M could be recovered from the MoAb-rosette-negative and the CALLA− fractions. Since none of the antibodies used during the rosette depletion reacted with CFU-G/M, the low number of colonies in the MoAb-rosette positive fractions strongly indicate that very little nonspecific loss of CFU-G/M occurred during rosette depletion.

## Discussion

The series of experiments described in this study was designed to purify and characterize normal hematopoietic cells that express CALLA from fetal liver and fetal bone marrow, where they represent a relatively small fraction of the total mononuclear cell population. A major reason for the success of the purification procedure was the initial MoAb-rosette depletion step, where R/M-SRBC were used to rosette leukocytes labeled with a mixture of MoAb against erythroid and myeloid differentiation antigens. In the subsequent rosette sedimentation, large numbers of erythroid and myeloid cells could be removed quickly and effectively. In this context, T9 antibody, which defines the transferrin receptor (20) was particularly helpful, because the majority of mononuclear cells in fetal liver suspensions, even after two F/H centrifugations, were normoblasts. In fetal bone marrow, the resulting rosette-negative cell suspensions were so enriched for CALLA+ cells that frequently these cells were >80% pure. Subsequently, further purification of CALLA+ cells was obtained either by cell sorting or by additional immune rosetting with J5 and J13 antibodies.

After purification, CALLA+ cells from both fetal liver and fetal bone marrow appeared to be a homogeneous population of medium-sized lymphocytes. However, with a series of monoclonal antibodies and immunofluorescence assays for intracytoplasmic-$\mu$ and TdT, we have been able to demonstrate that the phenotype of CALLA+ cells is strikingly heterogeneous. This was found particularly when markers for immature B cells were used, because only ~40% of the cells were positive for the B1 differentiation antigen and 15–30% expressed cyto-$\mu$ (Tables III and V). Furthermore, only a minority (5–25%) of the CALLA+ cells contained TdT (Table V). In contrast, virtually all CALLA+ cells expressed Ia, T10, and J2 antigens, and there were no major differences between cells isolated from fetal liver and fetal bone marrow. It should be noted, however, that some CALLA+ cells were also lost at each step of the purification procedure. For example, as shown in Table II, the MoAb-rosette-positive population contained 2% J5+ cells, and the MoAb-rosette-negative, J5-rosette-negative population contained 3% J5+ cells. As these CALLA+ cells comprised very small fractions of these populations, they could not be accurately

characterized. However, because these losses appeared to be nonspecific, we have assumed that the J5+ cells that were enriched and purified at each step are representative of the entire CALLA-positive population.

Earlier studies have demonstrated that leukemic cells from ~50% of patients with non-T ALL were reactive with B1 antibody (14). When expression of B1 antigen was correlated with expression of CALLA, it was found that expression of B1 was restricted to those tumor cells that were also CALLA+. Furthermore, recent studies using in vitro differentiation of ALL cells by phorbol diester showed that ALL cells which were CALLA+, B1− could be induced to express B1 surface antigen, whereas CALLA− ALL cells could not be induced to express B1 (26). Taken together, this analysis of leukemic cells suggested that the majority of CALLA+ non-T ALL cells were derived from the B cell lineage and led us to predict that CALLA would be expressed at an earlier point in B cell development than B1. The analysis of CALLA+ fetal hematopoietic cells indicates that a large fraction of these cells are indeed in early phases of B cell development. In fact, the phenotype of normal CALLA+ cells is almost identical to that of CALLA+ leukemic cells, and, as has been suggested previously (4, 5), these normal cells appear to be the normal counterpart of ALL cells. One major difference, however, is that only a small fraction of normal CALLA+ fetal lymphoid cells are TdT+, whereas almost all ALL cells contain this nuclear enzyme.

In addition to allowing a direct comparison of the surface markers of normal CALLA+ cells with leukemic cells, our purification of CALLA+ and CALLA− populations has allowed the identification of fetal lymphoid cells at different stages of maturation. It is evident that within the CALLA+ positive population at least three subsets can be identified. The first, and most differentiated, subset expresses both surface B1 and cyto-$\mu$, and therefore corresponds to a population that has previously been classified as "pre-B" cells (27, 28). In addition, these cells express Ia, J2, and T10 antigens (as do all CALLA+ cells), but few of these cells appear to be TdT+. The second, less mature, subset has an identical surface phenotype (CALLA+, Ia+, J2+, T10+, B1+) but does not express cyto-$\mu$ or TdT. These cells appear to be at an earlier stage of lymphoid differentiation, but the expression of B1 suggests that they have already been committed to the B cell lineage. In studies noted previously (26), ALL cells with this phenotype can be induced to express cyto-$\mu$ with phorbol diester. The third subset of CALLA+ cells expresses Ia, J2, and T10 but does not express B1. The lineage of these cells is not clearly established by their surface phenotype because none of these antigens are lineage specific. Although a significant fraction of these cells is TdT+, most are TdT−. It is likely that some cells within this group are very early B cells that will subsequently express B1 and then cyto-$\mu$ with further differentiation. However, the expression of CALLA is not restricted to B cells or B cell tumors because some T cell malignancies (T-ALL and lymphoblastic lymphoma) also express this antigen. It is therefore possible that some cells within this group may be T cell precursors, with yet-undefined surface markers, that will later migrate to the thymus where further differentiation will occur.

Within the CALLA− cell fraction that was first depleted of mature erythroid and myeloid cells by immune rosetting and then further purified by removal of CALLA+ cells, our results also indicate considerable heterogeneity. Firstly, Giemsa-stained smears demonstrated that this fraction consisted of both large blast cells and small- to medium-sized lymphocytes (Fig. 1). Approximately 60% were T10+, 55% were Ia+,

20% were J2+, and 15% were B1+ (Table IV). In addition, 35–50% of the CALLA− cells were TdT+. It therefore seems likely that this fraction contains very early lymphoid precursor cells in addition to almost all myeloid precursor cells (here, CFU-G/M). Phenotypically, these cells appear to represent the nonmalignant counterparts of undifferentiated CALLA− ALL cells. The heterogeneity of the CALLA− fraction is further emphasized by the small but significant number of B1+ cells within this population. Because we did not test for expression of surface Ig, it is possible that some of these CALLA−, B1+ cells are mature B cells. However, a small but significant percentage of CALLA− cells also express cyto-$\mu$, and it is therefore evident that not all pre-B are contained within the CALLA+ population. Recent clinical studies also support the conclusion that the most immature stem cells (both myeloid and lymphoid) do not express CALLA. In these studies, patients with CALLA+ ALL in second remission received ablative chemotherapy and total body irradiation followed by infusion of autologous bone marrow that had been depleted of CALLA+ cells (both normal and leukemic) by in vitro treatment with J5 antibody and C (29). All patients thus far have been engrafted with antibody-treated bone marrow, and two patients who have been followed for >1 yr have demonstrated complete reconstitution of all myeloid and lymphoid populations.

Earlier studies using CALLA+ leukemia cells and cell lines have shown that J5 antibody induces antigenic modulation of cell surface antigen (8). This phenomenon has had important implications for the passive serotherapy of patients with ALL because J5 antibody infusions lead to loss of CALLA expression on malignant cells without impeding their growth (9). The data presented in Fig. 3 and Table VII demonstrate that modulation of CALLA by J5 antibody is a physiologic phenomenon that occurs on normal CALLA+ cells as well. Thus, whereas CALLA modulation may be a mechanism whereby leukemic cells may become resistant to antibody-mediated lysis, the modulation of CALLA on fetal cells may be indicative of a normal functional property of this cell surface glycoprotein. Other surface molecules that undergo antigenic modulation such as surface Ig (30), C3b receptor (31), and T3 antigen (32) appear to have specific membrane receptor-like functions; CALLA modulation may therefore be analogous to the phenomenon of "down-regulation" in which expression of cell surface receptor is decreased in the presence of excess ligand or hormone (33, 34).

## Summary

Fetal hematopoietic cells that express the common acute lymphoblastic leukemia antigen (CALLA) were purified from both fetal liver and fetal bone marrow by immune rosetting with sheep erythrocytes coated with rabbit anti-mouse immunoglobulin and by fluorescence-activated cell sorting. Dual fluorescence techniques disclosed that these cells were heterogenous with respect to the expression of a series of differentiation and activation antigens defined by monoclonal antibodies. Thus, whereas all CALLA+ cells were Ia+ and expressed two activation antigens, J2 and T10, only 30–50% expressed B1 antigen. Furthermore, using methanol-fixed cells, it could be shown that ~20% contained intracytoplasmic $\mu$ chains (cyto-$\mu$) and that ~15% were positive for the terminal transferase enzyme (TdT) marker. The CALLA+ fetal cells thus closely resemble the childhood acute lymphoblastic leukemia cell with respect to surface marker phenotype. A population of CALLA− cells devoid of mature erythroid and myeloid surface markers was found to contain higher numbers of TdT+ cells but lower numbers of cyto-$\mu$, B1, and Ia+ cells than the CALLA+ subset. In

vitro analysis of normal, purified CALLA+ cells demonstrated that incubation at 37°C with J5 monoclonal antibody specific for CALLA resulted in the specific modulation of surface antigen. Similar results have previously been obtained with CALLA+ tumor cells. Although phenotypic analysis of CALLA+ cells suggests that these cells are relatively immature lymphoid cells, CALLA+ cells do not appear to contain either myeloid precursor cells (CFU-G/M) or the earliest lymphoid stem cells.

We thank Lori Palley for technical assistance and Pearl Leavitt for help in preparing the photomicrographs.

*Received for publication 7 July 1982 and in revised form 27 August 1982.*

## References

1. Greaves, M. F., G. Brown, N. T. Rapson, and T. A. Lister. 1975. Antisera to acute lymphoblastic leukemia cells. *Clin. Immunol. Immunopathol.* **4:**67.
2. Ritz, J., J. M. Pesando, J. Notis-McConarty, H. Lazarus, and S. F. Schlossman. 1980. A monoclonal antibody to human acute lymphoblastic leukemia antigen. *Nature (Lond.).* **283:**583.
3. Ritz, J., L. M. Nadler, A. K. Bhan, J. Notis-McConarty, J. M. Pesando, and S. F. Schlossman. 1981. Expression of common acute lymphoblastic leukemia antigen (CALLA) by lymphomas of B cell and T cell lineage. *Blood.* **58:**648.
4. Janossy, G., F. J. Bollum, K. F. Bradstock, A. McMichael, N. Rapson, and M Greaves. 1979. Terminal transferase bone marrow cells exhibit the antigen phenotype of common acute lymphoblastic leukemia. *J. Immunol.* **123:**1525.
5. Greaves, M., D. Delia, G. Janossy, N. Rapson, J. Chessels, M. Woods, and G. Prentice. 1980. Acute lymphoblastic leukemia associated antigen. IV. Expression on nonleukemic "lymphoid" cells. *Leukemia Res.* **4:**15.
6. Pesando, J. M., J. Ritz, H. Levine, C. Terhorst, H. Lazarus, and S. F. Schlossman. 1980. Human leukemia-associated antigen: relation to a family of surface glycoproteins. *J. Immunol.* **124:**2794.
7. Metzgar, R. S., M. J. Borowitz, N. H. Jones, and B. L. Dowell. 1981. Distribution of common acute lymphoblastic leukemia antigen in nonhematopoietic tissues. *J. Exp. Med.* **154:**1249.
8. Ritz, J., J. M. Pesando, J. Notis-McConarty, and S. F. Schlossman. 1980. Modulation of human acute lymphoblastic leukemia antigen induced by monoclonal antibody in vitro. *J. Immunol.* **125:**1506.
9. Ritz, J., J. M. Pesando, S. E. Sallan, L. A. Clavell, J. Notis-McConarty, P. Rosenthal, and S. F. Schlossman. 1981. Serotherapy of acute lymphoblastic leukemia with monoclonal antibody. *Blood.* **58:**141.
10. Pesando, J. M., J. Ritz, H. Lazarus, K. J. Tomaselli, and S. F Schlossman. 1981. Fate of common acute lymphoblastic leukemia antigen during modulation by specific antibody. *J. Immunol.* **126:**540.
11. Hercend, T., L. M. Nadler, J. M. Pesando, E. L. Reinherz, S. F. Schlossman, and J. Ritz. 1981. Expression of a 26,000 dalton glycoprotein on activated human T cells. *Cell. Immunol.* **64:**192.
12. Nadler, L. M., P. Stashenko, R. Hardy, J. M. Pesando, E. J. Yunis, and S. F. Schlossman. 1981. Monoclonal antibodies defining serologically distinct HLA-D/DR related Ia antigens in man. *Human Immunol.* **1:**77.
13. Stashenko, P., L. M. Nadler, R. Hardy, and S. F. Schlossman. 1980. Characterization of a human B lymphocyte specific antigen. *J. Immunol.* **125:**1678.
14. Nadler, L. M., P. Stashenko, J. Ritz, R. Hardy, J. M. Pesando, and S. F. Schlossman. 1981. A unique cell surface antigen identifying lymphoid malignancies of B cell origin. *J. Clin. Invest.* **67:**134.

15. Todd, R. F., L. M. Nadler, and S. F. Schlossman. 1981. Antigens on human monocytes identified by monoclonal antibodies. *J. Immunol.* **126:**1435.
16. Todd, R. F., A. van Agthoven, S. F. Schlossman, and C. Terhorst. 1982. Structural analysis of differentiation antigens Mo1 and Mo2 on human monocytes. *Hybridoma.* **3:**329.
17. Griffin, J. D., J. Ritz, L. M. Nadler, and S. F. Schlossman. 1981. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. *J. Clin. Invest.* **68:**932.
18. Reinherz, E. L., R. E. Hussey, and S. F. Schlossman. 1980. A monoclonal antibody blocking human T cell function. *Eur. J. Immunol.* **10:**758.
19. Reinherz, E. L., P. C. Kung, G. Goldstein, R. H. Levy, and S. F. Schlossman. 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T lineage. *Proc. Natl. Acad. Sci. U. S. A.* **58:**954.
20. Sutherland, R., D. Delia, C. Schneider, R. Newman, J. Kemshead, and M. Greaves. 1981. Ubiquitous cell surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. *Proc. Natl. Acad. Sci. U. S. A.* **78:**4515.
21. Hercend, T., J. Ritz, S. F. Schlossman, and E. L. Reinherz. 1981. Comparative expression of T9, T10 and Ia antigens on activated human T cell subsets. *Human Immunol.* **3:**247.
22. Meuer, S. C., S. F. Schlossman, and E. L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions. *Proc. Natl. Acad. Sci. U. S. A.* **79:**4395.
23. Griffin, J. D., R. P. Beveridge, and S. F. Schlossman. 1982. Isolation of myeloid progenitor cells from peripheral blood of chronic myelogenous leukemia patients. *Blood.* **60:**30.
24. Bollum, F. J. 1979. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. *Blood.* **54:**1203.
25. Clavell, L. A., J. M. Lipton, R. C. Bast, M. Kudisch, J. M. Pesando, S. F.Schlossman, and J. Ritz. 1981. Absence of common ALL antigen on bi-potent myeloid, erythroid, and granulocyte progenitors. *Blood.* **58:**333.
26. Nadler, L. M., J. Ritz, M. P. Bates, E. K. Park, K. C. Anderson, S. E. Sallan, and S. F. Schlossman. 1982. Induction of human B cell antigens in non-T cell acute lymphoblastic leukemia. *J. Clin. Invest.* **70:**433.
27. Pearl, E. R., L. B. Vogler, A. J. Okos, W. M. Christ, A. R. Lawton, and M. D. Cooper. 1978. B lymphocytic precursors in human bone marrow: an analysis of normal individuals and patients with antibody deficiency states. *J. Immunol.* **120:**1169.
28. Owen, J. J. T. 1980. B cell development. *Prog. Immunol.* **4:**303.
29. Ritz, J., S. E. Sallan, R. C. Bast, J. M. Lipton, L. A. Clavell, M. Feeney, T. Hercend, D. G. Nathan, and S. F. Schlossman. 1982. Autologous bone marrow transplantation in CALLA positive acute lymphoblastic leukemia following in vitro treatment with J5 monoclonal antibody and complement. *Lancet.* **II:**60.
30. Knopf, P. M., A. Destree, and R. Hyman. 1973. Antibody-induced changes in expression of an immunoglobulin surface antigen. *Eur. J. Immunol.* **3:**251.
31. Fearon, D. T., I. Kaneko, and G. G. Thomson. 1981. Membrane distribution and adsorptive endocytosis by C3b receptors on human polymorphonuclear leukocytes. *J. Exp. Med.* **153:**1615.
32. Reinherz, E. L., S. C. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell.* **30:**735.
33. Baldwin, D., M. Prince, S. Marshall, P. Davies, and J. M. Olefsky. 1980. Regulation of insulin receptors: evidence for involvement of an endocytic internalization pathway. *Proc. Natl. Acad. Sci. U. S. A.* **77:**5975.
34. Pastan, I. H., and M. C. Willingham. 1981. Journey to the center of the cell: role of the receptosome. *Science (Wash. D. C.).* **214:**504.