

Absence of Common ALL Antigen on Normal Bipotent Myeloid, Erythroid, and Granulocyte Progenitors

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The presence of the common acute lymphoblastic leukemia antigen (CALLA) on leukemic cells from the great majority of patients with non-T-cell acute lymphoblastic leukemia and chronic myelogenous leukemia in blast crisis suggests that CALLA could be a differentiation antigen expressed by normal lymphoid and myeloid stem cells. Treatment with a murine monoclonal anti-CALLA antibody

and complement lysed CALLA-positive leukemic cells quantitatively, whereas similar treatment of nucleated cells from peripheral blood and bone marrow failed to affect the expression, in semisolid culture, of CFU-G/E, BFU-E, CFU-E, or CFU-C. These data suggest that CALLA is not a normal differentiation antigen of the myeloid bipotent cell or its committed progenitors.

PREVIOUS STUDIES by Greaves¹ and others²⁻⁶ have demonstrated the existence of an antigen associated with cells from many patients with acute lymphoblastic leukemia (ALL) and some patients with chronic myelocytic leukemia (CML) in blast crisis. A monoclonal hybridoma antibody to this common ALL antigen (CALLA) has been developed and characterized by Ritz.⁷ The exact nature of a CALLA-positive cell is unknown, but it has been proposed that normal lymphocyte precursor or pluripotent stem cells express CALLA.⁸ Janossy,⁸ using rabbit anti-CALLA, has presented data suggesting that small cells of lymphoid morphology in normal bone marrow that express CALLA are not CFU-C. With the development of semisolid culture techniques for short-term cultures of bipotential myeloid⁹ and immature erythroid progenitors, the presence of CALLA on the surface of these cells can now be explored.

Clonal assays of granulocyte and erythroid progenitors in humans are well described.¹⁰⁻¹³ Johnson and Metcalf in the mouse¹⁴ and Fauser and Messner in man^{9,15} have recently reported a semisolid culture system for the expression of mixed colonies containing granulocyte-macrophages and erythroblasts, the latter capable of high fetal hemoglobin production, indicating the immature nature of this progenitor.¹⁵ Data presented in this study show that CALLA is not a normal differentiation antigen present on the surface of this multipotential immature myeloid progenitor. The absence of the antigen is noted as well on the more mature committed erythroid-granulocyte-macrophage progenitors.

MATERIALS AND METHODS

Donors

Bone marrow and peripheral blood from four healthy volunteers was collected, after informed consent, in syringes containing preservative-free heparin. Care was taken to aspirate no more than 2 ml of marrow, the yield of which was approximately $40 \pm 10 \times 10^6$ nucleated cells after separation by Ficoll-Hypaque centrifugation.

CALLA-positive leukemic cells were obtained from the peripheral blood of a patient with ALL. After Ficoll-Hypaque separation, this population contained >99% lymphoblasts as determined by morphological criteria.

Antibody Treatment

A monoclonal cytotoxic IgG antibody, reactive with human CALLA, termed J-5, prepared by hybridoma techniques¹⁶ as recently described⁷ was utilized. Its activity was established by serial dilution of the murine ascites fluid into which it was secreted.⁷ A 1:500 dilution of the murine ascites fluid in S-MEM (Grand Island Biological Company, Grand Island, N.Y.) medium with 10 mM Hepes buffer and 5% fetal calf serum (FCS) (Flow Labs, Rockville, Md.) was utilized. A similar diluted nonspecific murine ascites, designated J-0, lacking specific antibody activity, was used as a control.

Mononuclear cells isolated by Ficoll-Hypaque centrifugation at a concentration of $20-40 \times 10^6$ cells/ml were incubated with J-5 or J-0 at 4°C for 30 min. Then, 0.15 ml of fresh frozen rabbit serum (lot C1192108, Grand Island Biological Company) was added as a complement source and the mixture incubated at 37°C for 1 hr with gentle shaking. After incubation, prior to plating in culture, the cells were washed 3 times in alpha medium minus nucleosides (α . Grand Island Biological Company) plus 5% FCS.

CALLA-positive lymphoblasts were labeled by incubation for 30 min with sodium ⁵¹chromate ($0.2 \text{ mCi}/2 \times 10^7$ cells). After 3 washes to remove excess ⁵¹Cr, labeled lymphoblasts (10^6 cells) were mixed with normal bone marrow (10^8 cells) and treated for three 30-min periods with antibody and complement (C'). Lysis of

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lymphoblasts was calculated from the ^{51}Cr counts associated with cells following treatment with J-5 and C', J-0 and C', or with medium that lacked these reagents. Percent lysis of cells was calculated by comparison with ^{51}Cr counts remaining after freezing and thawing of labeled cells 3 times prior to treatment.

Clonal Assays

CFU-E and BFU-E clonal assay in plasma clot. Mononuclear cells at a concentration of $5 \times 10^5/\text{ml}$ or $2 \times 10^6/\text{ml}$ obtained from bone marrow or peripheral blood, respectively, were added in 0.1 ml of α - and 5% FCS to 0.8 ml of the erythropoietin-dependent plasma clot system of McLeod and coworkers,¹⁷ as modified by Clarke and Housman¹³ and Nathan and co-workers.¹⁸ Erythropoietin (Connaught Step III, Connaught Laboratories, Willowdale, Ontario, Canada) at a specific activity of approximately 5 IU/mg protein was used at a final concentration of 2 IU/ml plasma clot. The cultures were incubated at 37°C in 100% humidity and 5% CO_2 . The clots were fixed and stained. CFU-E and BFU-E colony enumeration was done on day 7 and days 11–14, respectively, as previously described.¹⁸

CFU-G/E, BFU-E, and CFU-C clonal assays in methylcellulose. The appropriate nucleated cell number, 2×10^5 cells/ml for bone marrow and 4×10^5 cells/ml for peripheral blood, was mixed into 30% FCS, with and without 10% PHA-LCM,¹⁹ Dulbecco's modified Eagle's medium (Grand Island Biological Company), and methylcellulose as viscous support to yield a concentration of 0.9% (w/v).⁹ Erythropoietin at a final concentration of 1.5 IU/ml was added on day 4 of the incubation. The cultures were incubated at 37°C in 100% humidity and 5% CO_2 . CFU-C colonies were scored on day 14 and BFU-E and CFU-G/E colonies on day 18 at 40 \times magnification using a dissecting microscope (Olympus Trinocular stereo microscope, Olympus Corporation of America, New Hyde Park, N.Y.). CFU-C were identified by their lack of color and relatively diffuse pattern. BFU-E were easily recognized by their red color and tightly packed colonies. BFU-E were defined as erythroid colonies having a minimum of three subcolonies, the range being from 3 to 22 subcolonies. CFU-G/E were recognized by their heterogeneity with respect to the distribution of cells with a red color typical of hemoglobin. Cells in some areas appeared predominantly red, while cells in other areas of the same colony lacked this typical feature of erythropoietic cells. All CFU-G/E colonies were removed from culture, mounted on slides, and stained appropriately as previously described⁹ to confirm the coexistence of erythroid and granulocytic cells, respectively, in single colonies. All of the colonies were not stained for the presence of megakaryocytes; therefore, the progenitors are designated CFU-G/E⁹ rather than CFU-GEMM.¹⁵

PHA-LCM. Media conditioned by peripheral blood leukocytes of normal individuals in the presence of 1% phytohemagglutinin was prepared as previously described.¹⁹

RESULTS

Bone Marrow Progenitors

Granuloerythropoietic (CFU-G/E) colonies in methylcellulose culture. Table 1 shows the number of CFU-G/E colonies/ 10^6 nucleated cells plated after treatment with either J-0 or J-5 plus complement. Although the number of colonies are necessarily small, there was no difference between the two groups. In addition, colony size and morphology were identical in the two groups. The presence of 10% PHA-LCM as previously reported⁹ was required for colony expression.

Table 1. Effect of Anti-CALLA Antibody and Complement Treatment on Bone Marrow and Peripheral Blood Derived Granuloerythropoietic (CFU-G/E) Colony Expression in Methylcellulose Culture

| | Experiment A | | Experiment B | | Experiment C* | |
|---|--------------|-----|--------------|-----|---------------|-----|
| Bone marrow nucleated cells | | | | | | |
| Treatment | J-0 | J-5 | J-0 | J-5 | J-0 | J-5 |
| CFU-G/E colonies/ 10^6 cells plated | 10 | 9 | 13 | 12 | 12 | 10 |
| Peripheral blood nucleated cells | | | | | | |
| Treatment | J-0 | J-5 | J-0 | J-5 | | |
| CFU-G/E colonies/ 10^6 cells plated | 9 | 8 | 12 | 13 | | |

*In this experiment, the cell mixture consisted of 10^6 CALLA-positive lymphoblasts/ 10^8 normal bone marrow cells. One-hundred percent lysis of CALLA-positive cells was obtained after three antibody and complement treatments. The residual cells were then cultured.

Burst-forming units erythroid (BFU-E) and colony-forming units culture (CFU-C) in methylcellulose culture. Table 2 shows the number of BFU-E and CFU-C derived colonies after control or anti-CALLA antibody plus complement treatment to be the same. Some enhancement of BFU-E formation after the addition of 10% PHA-LCM was noted in experiment A, but not in experiment B. The CFU-C colony-stimulating activity of the PHA-LCM is present in experiments A and B. The size, morphology, as well as the number of colonies were no different in the antibody-treated and control groups.

Burst-forming units erythroid (BFU-E) and colony-forming units erythroid (CFU-E) in plasma clot. Table 3 compares BFU-E and CFU-E colony expression in plasma clot after either J-0 or J-5 and complement treatment. Comparable numbers of progenitor colonies were obtained after treatment with either anti-CALLA antibody or the control ascites. Once again, colony size and morphology were similar in the two groups. Colony expression from BFU-E and CFU-E was equal after incubation with either J-0, the control, or J-5, the antibody.

Peripheral Blood Progenitors

Granuloerythrocytic (CFU-G/E) colonies in methylcellulose. Table 1 shows the number of CFU-G/E colonies per 10^6 nucleated cells from peripheral blood plated after treatment with either J-0 or J-5 and complement. No difference in number, size, or morphology of colonies was observed between the two groups.

Burst-forming units erythroid (BFU-E) and colony-forming units in culture (CFU-C) in methylcellulose. Table 2 demonstrates no effect of either J-0 or J-5 and complement treatment on the expression of

Table 2. Effect of Anti-CALLA Antibody and Complement Treatment on Bone Marrow and Peripheral Blood Derived BFU-E and CFU-C Colony Expression in Methylcellulose Culture

| | Experiment A | | | | Experiment B | | | | Experiment C* | |
|--|--------------|---------|---------|---------|--------------|---------|---------|----------|---------------|---------|
| Bone marrow nucleated cells | | | | | | | | | | |
| Treatment | J-0 | J-0 | J-5 | J-5 | J-0 | J-0 | J-5 | J-5 | J-0 | J-5 |
| Additive | — | PHA-LCM | — | PHA-LCM | — | PHA-LCM | — | PHA-LCM | PHA-LCM | PHA-LCM |
| BFU-E colonies/ 2 × 10 ⁵ cells plated | 24 ± 9 | 43 ± 9 | 14 ± 4 | 39 ± 5 | 84 ± 13 | 97 ± 20 | 88 ± 21 | 95 ± 9 | 23 ± 4 | 30 ± 3 |
| CFU-C colonies/ 2 × 10 ⁵ cells plated | 13 ± 4 | 80 ± 2 | 10 ± 1 | 85 ± 7 | 70 ± 14 | 118 ± 3 | 83 ± 5 | 108 ± 13 | | |
| Peripheral blood nucleated cells | | | | | | | | | | |
| Treatment | J-0 | J-0 | J-5 | J-5 | J-0 | J-5 | | | | |
| Additive | — | PHA-LCM | — | PHA-LCM | — | — | | | | |
| BFU-E colonies/ 4 × 10 ⁵ cells plated | 55 ± 33 | 60 ± 7 | 58 ± 8 | 65 ± 10 | 67 ± 11 | 68 ± 8 | | | | |
| CFU-C colonies/ 4 × 10 ⁵ cells plated | 49 ± 17 | 50 ± 14 | 50 ± 15 | 40 ± 7 | 34 ± 10 | 33 ± 7 | | | | |

*In this experiment, the cell mixture consisted of 10⁶ CALLA-positive lymphoblasts per 10⁸ normal bone marrow cells. One-hundred percent lysis of CALLA-positive cells was obtained after three antibody and complement treatments. The residual cells were then cultured.

BFU-E and CFU-C colonies from peripheral blood mononuclear cells. Growth characteristics were no different for the two groups.

Lysis of leukemia cells in the presence of normal marrow. A single incubation with J-5 and complement lysed 87% of leukemic lymphoblasts, while three incubations with J-5 and complement lysed >99% of leukemic lymphoblasts, but failed to affect colony expression from bone-marrow-derived CFU-G/E and BFU-E, as shown by representative studies described in Tables 1 and 2.

DISCUSSION

The utilization of specific antibodies for eradication of leukemic lymphoblasts from a remission marrow prior to autologous transplantation presupposes that the antigenic specificity of the antibody spares stem cells that will reconstitute the patient. J-5 anti-CALLA antibody fails to react with normal hematopoietic tissue as judged by indirect immunofluorescence. The presence of CALLA in blast crisis CML has, however, suggested that CALLA could represent a normal myeloid differentiation antigen. Using a conventional heteroantiserum, Greaves²⁰ has reported

the detection of CALLA-positive cells in regenerating marrow, consistent with the possibility that the CALLA-positive cell may be a lymphocyte precursor or stem cell. The current scheme of stem cell differentiation describes lymphoid and myeloid progenitors derived from a common bipotential stem cell. The myeloid pluripotential cell gives rise to the more mature committed progenitors of the erythrocyte, granulocyte, macrophage, megakaryocyte, and eosinophil lines. The presence of these cells in marrow is inferred from cultures of marrow cells in semisolid media, which reveal the growth of unique colonies derived from CFU-G/E (myeloid bipotential cells), BFU-E (immature erythroid progenitors), CFU-E (mature erythroid progenitors), and CFU-C (granulocyte and macrophage progenitors). The same number of colonies with comparable size and morphology was observed in the CFU-G/E, BFU-E, CFU-E, and CFU-C assays, respectively, after treatment with the anti-CALLA antibody J-5 and complement as with the nonspecific ascites control J-0. Most important is the fact that the colonies derived from normal progenitors were expressed in culture even in experiments in which CALLA-positive leukemic cells admixed with

Table 3. Effect of Anti-CALLA Antibody and Complement Treatment on Bone-Marrow-Derived BFU-E and CFU-E Colony Expression in Plasma Clot Culture

| | Experiment A | | Experiment B | | Experiment C | |
|--|--------------|----------|--------------|----------|--------------|---------|
| Treatment | J-0 | J-5 | J-0 | J-5 | J-0 | J-5 |
| BFU-E colonies/10 ⁵ cells plated | 93 ± 23 | 70 ± 11 | 9 ± 3 | 11 ± 2 | 19 ± 6 | 32 ± 6 |
| CFU-E colonies/10 ⁵ cells plated | 399 ± 51 | 308 ± 79 | 195 ± 33 | 227 ± 38 | 139 ± 45 | 139 ± 7 |

normal marrow were recognized by the J-5 antibody and lysed with complement. This suggests the absence of CALLA in the normal bone marrow and peripheral blood myeloid pluripotent cells and their committed progenitors. This does not rule out the presence of CALLA on a more primitive myeloid cell for which no *in vitro* assay currently exists. The appearance of CALLA on the surface of cells during lymphoid ontogeny is not excluded by our experiments, in part because appropriate clonal assays for normal lymphoid cells are not yet available. The high specific-

ity of complement-mediated cell lysis using this monoclonal hybridoma antibody, J-5, makes it a powerful potential tool for the *in vitro* elimination of CALLA-positive lymphoblasts from marrow prior to autologous bone marrow transplantation. Such transplantations will provide better evidence for CALLA specificity.

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