A SELECTIVE CULTURE SYSTEM FOR GENERATING TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE–POSITIVE (TdT^+) LYMPHOID PRECURSOR CELLS IN VITRO

I. Description of the Culture System

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In previous studies, we and others have demonstrated that the enzyme terminal deoxynucleotidyl transferase (TdT)[1] normally is restricted to primitive lymphoid cells, most certainly of the T cell series and possibly of the B cell series as well (reviewed in 1–3). Our studies have also suggested that TdT^+ bone marrow cells or their immediate precursors (pre-TdT^+ cells) may serve as the transformable target cells for the Gross leukemia virus (4), and that abnormalities in the development of TdT^+ bone marrow cells and thymocytes are among the earliest lymphoid defects to occur in mutant strains of autoimmune NZB/W, BXSB, and SJL/J mice.[2] Other authors (5–7) have postulated that TdT may itself play an important role in the antigen-independent development of lymphocytes, either in the generation of immunological diversity and/or in the elimination of self-reactive clones of immature lymphocytes. Thus, it is likely that further studies of TdT^+ bone marrow cells would provide valuable information concerning the early stages of normal and abnormal lymphocyte development.

Progress in the study of the development and differentiation of TdT^+ cells, and of the antigen-independent phase of lymphopoiesis generally, has been hampered by the absence of a selective in vitro system for culturing lymphoid stem and progenitor cells. Earlier (8), we demonstrated that small numbers (~2%) of mouse TdT^+ bone marrow cells could be generated in modified Dexter-

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[1] *Abbreviations used in this paper:* BMX, bone marrow extract; CFU-S, colony-forming unit–spleen; ChXFBS, charcoal-extracted fetal bovine serum; CM, conditioned medium; FACS, fluorescence-actived cell sorter; FBS, fetal bovine serum; FGF, fibroblast growth factor; GM-CFC, granulocyte/macrophage in vitro colony-forming cell; GM-CSF, granulocyte/macrophage colony-stimulating factor; GP-BMX, guinea pig BMX; M-BMX; mouse BMX; PBS, phosphate-buffered saline; PGE_1, prostaglandin E_1; R-BMX, rat BMX; SCM, rat spleen conditioned medium; TdT, terminal deoxynucleotidyl transferase.

[2] Whittum, J., I. Goldschneider, D. L. Greiner, and R. Zurier. Developmental abnormalities of terminal deoxynucleotidyl transferase–positive bone marrow cells and thymocytes in New Zealand mice: effects of prostaglandin E_1. Submitted for publication.

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type cultures for at least 7 wk. Such cultures also contained colony-forming unit–spleen (CFU-S) and myeloid and erythroid progenitor cells. In the present study, we describe a primary xenogeneic culture system in which rat TdT^+ bone marrow cells are selectively generated on feeder layers of mouse bone marrow cells under conditions that do not support the survival of CFU-S or nonlymphoid progenitor cells. The cultured TdT^+ bone marrow cells do not cause leukemia, are indistinguishable phenotypically from their normal counterparts in vivo, and can be generated with equal facility from the bone marrow of normal and congenitally athymic (*nu/nu*) rats.

Materials and Methods

Animals

3–5-wk-old male (Lewis × DA)F_1 (LDA F_1) rats and (C57BL/6J × A/Thy-1.1)F_1 (B6A F_1) mice from our breeding colonies were used in most of the experiments. Other experimental animals used in some experiments included: F344, Buffalo (BUF), Albany (ALB), M520, and NIH-r*nu* (*nu/nu* and *nu/+*) strain rats; and C57BL/6J, C57BL/10, A/J, DBA/2J, NZW, and NZB strain mice.

Culture Medium, Hormones, and Growth Factors

RPMI 1640 (Flow Laboratories, McLean, VA) contained 0.015 M Hepes buffer and 50 μg/ml gentamycin sulfate (Schering Co., Kenilworth, NJ).

Aliquots of ~500 ml of fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY) were mixed with 1 mg/ml dextran (average mol wt, 40,000) and 10 mg/ml Norit A activated charcoal (Sigma Chemical Co., St. Louis, MO) in a gyratory water bath shaker for 30 min at 56°C. The activated charcoal was removed by centrifugation (12,000 *g* for 10 min) at 4°C and the charcoal-extracted FBS (ChXFBS) was sterilized by membrane filtration. This treatment has been shown to remove >90% of the steroid hormones and ~30% of the thyroid hormone, but <2% of the total protein from serum (9). Additionally, defined FBS (HyClone) was purchased from Sterile Systems, Inc., Logan, UT. This FBS had very low levels of endotoxin and steroid hormones, obviating the need for charcoal extraction. All of the above sera were stored at −70°C until immediately before use.

Fibroblast growth factor (FGF) (Collaborative Research Inc., Waltham, MA) was reconstituted in phosphate-buffered saline (PBS) at a stock concentration 1,000-fold higher than the final concentration and was stored at −70°C. Hydrocortisone, prostaglandin E_1 (PGE_1), estradiol-17β, progesterone, aldosterone, and testosterone (Sigma Chemical Co.), and dexamethasone (Organon, Inc., W. Orange, NJ) were dissolved in 95% ethanol at a concentration of 1×10^{-2} M and were stored at −20°C.

Bone marrow extracts (BMX) were prepared by sonicating (Biosonik: Bronwill Scientific, Rochester, NY) plugs of femoral bone marrow in cold PBS at a ratio of 1:3. The sonicates were centrifuged (480 *g* for 15 min) at 4°C to remove tissue fragments, and then centrifuged at 25,000 *g* for 20 min at 4°C. The supernatants were sterilized using an 0.2-μm filter unit (Nalge Co., Nalgene Labware Div., Rochester, NY). The protein contents of the supernatants were measured by the Coomassie Blue colorimetric assay (10).

Preparation of Feeder Layers

Bone marrow cells from the femur and tibia of 6–12 animals were aseptically collected into a sterile centrifuge tube by perfusing the shaft with culture medium. A single-cell suspension was made by repetitive pipetting. ~1×10^7 nucleated cells in 2 ml RPMI 1640 supplemented with 10% FBS were placed in 35-mm diameter, flat-bottom plastic culture dishes (Costar, Cambridge, MA) and incubated at 37°C in 5% CO_2 in air. The medium was changed on day 7. By day 10 the culture wells contained confluent layers of tightly adherent cells.

Spleen and thymus were minced finely with sterile scalpels in RPMI 1640 supplemented with 10% FBS. The organ fragments were washed in the medium and cultured in 60-mm diameter plastic tissue culture dishes (Corning Glass Works, Corning, NY). The culture medium was changed once a week until a confluent adherent layer was obtained.

Culture of Bone Marrow Cells

Nonadherent cells were removed from the confluent feeder layers by washing with fresh medium. The cultures, prepared in triplicate, were then charged with single-cell suspensions of bone marrow (1×10^3 to 1×10^6 cells/ml) in RPMI 1640 supplemented with 30% defined, undefined, or ChXFBS.

Antisera

The following mouse anti-rat monoclonal antibodies were purchased: W3/13 (pan–T cell), W3/25 (helper/inducer T cell), OX3 (Ia, polymorphic), OX4 (Ia, common determinant), OX8 (suppressor/cytotoxic T cell), and OX19 (pan–T cell) (11) (Accurate Chemical and Scientific Corp., Westbury, NY); Thy-1.1 (New England Nuclear, Boston, MA). Rat monoclonal antibodies to the rat RT-7^a (A. R. T.-1^a) pan–T cell alloantigen, the RT-6^a (A. R. T.-2^a) peripheral T cell alloantigen, and the A. R. T.-3 (CS1.21) cortical thymocyte alloantigen were produced as previously described (12). Alloantiserum to the RT-1 rat major histocompatibility antigen was prepared by immunizing Lewis strain rats with lymph node and spleen cells from DA strain rats (13). After absorption with LEW rat erythrocytes and B6A F_1 mouse spleen cells, the antiserum reacted with LDA F_1 rat bone marrow cells but not with B6A F_1 mouse bone marrow cells. Alloantiserum to the H-2 mouse major histocompatibility antigen (H-2^b) was a kind gift from Dr. Herbert Freedman (State University of New York, Downstate Medical Center, Brooklyn). Affinity column-purified IgG $F(ab')_2$ rabbit antibodies to homogeneous calf TdT was obtained from Dr. F. Bollum (Uniformed Services University of the Health Sciences, Bethesda, MD) (14, 15). Fluorescein isothiocyanate (FITC)–goat IgG $F(ab')_2$ anti-mouse IgG, TRITC–goat IgG $F(ab')_2$ anti-mouse IgG, TRITC–goat IgG $F(ab')_2$ anti-rat IgG, FITC–goat IgG $F(ab')_2$ anti-rabbit IgG, and goat IgG anti-rat IgM were purchased from Cappel Laboratories (Cochranville, PA).

Immunofluorescence

Cell surface antigens. $\sim 1.5 \times 10^6$ nucleated cells were washed in RPMI 1640 containing 20 mM sodium azide, incubated in suspension with the primary antiserum for 15 min at 4°C, washed, and developed for immunofluorescence with TRITC- or FITC-conjugated goat IgG $F(ab')_2$ against the primary antiserum. The percentage of positive cells was determined by fluorescence microscopy.

TdT. $\sim 1.2 \times 10^5$ nucleated cells were smeared onto glass slides with a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA), air dried, and fixed in cold absolute methanol for 15 min at 4°C. The smears were then incubated with 10 μl of rabbit anti-calf TdT (30 μg/ml) for 15 min at room temperature, washed, and developed with FITC-goat IgG $F(ab')_2$ anti-rabbit IgG (15). Double immunofluorescence for surface antigens and TdT was performed by staining viable cells in suspension before smearing and fixing the cells (16).

Cell Separation and Analysis on the Fluorescence-activated Cell Sorter (FACS)

Cells were analyzed and sorted on the FACS (FACS IV; B-D FACS Systems, Sunnyvale, CA) according to their relative low angle light scatter (1.5–15°) and relative fluorescence intensity for surface antigens as described previously (17).

CFU-S Assay

The in vivo CFU-S assay for pluripotent hematopoietic stem cells (18) was performed in irradiated (750 rad) 4–6-wk-old LDA F_1 strain rats as described previously (17). 1×10^6 freshly harvested rat bone marrow cells were injected as a positive control; 1 ml of

RPMI 1640 was injected as a negative control. The macroscopically visible colonies were counted 10 d after the injection.

GM-CFC Assay

The in vitro colony-forming cell assay for rat and mouse myeloid progenitor cells (GM-CFC) was performed as described previously (17). In the present study the agar medium was a mixture of 1 part 3% bacto-difco agar in 9 parts of McCoy's 5A medium supplemented with 15% heat-inactivated rat serum or FBS, 0.015 M Hepes buffer, and 50 μg/ml gentamycin sulfate (Schering Co.).

Colony formation was stimulated by addition of 0.1 ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) to each plate. GM-CSF was obtained from rat spleen conditioned medium (SCM) induced with pokeweed mitogen (17). Conditioned medium from cultures of rat TdT^+ cells (TdT-CM) was also tested for GM-CSF activity. Culture medium was harvested on day 10 after seeding mouse bone marrow feeder layers with rat bone marrow cells from primary or long-term cultures.

Autoradiography

Cultured rat bone marrow cells were pulsed for 1 h with 1 μCi per well of [^{3}H]thymidine (New England Nuclear Co.). The cells were washed, smeared on glass slides, fixed, developed for TdT immunofluorescence, extracted with 5% TCA, and processed for autoradiography as described previously (8). Slides treated with DNase (Sigma Chemical Co.) for 30 min before TCA treatment served as controls.

Characterization of TdT

The structure, molecular weight, and specific activity of TdT in cell extracts was kindly determined by Dr. F. J. Bollum (Uniformed Services University of the Health Sciences, Bethesda, MD) as described elsewhere (19).

Leukemia Cells

Two thymus-dependent, TdT^+ leukemia cell types were used. G-1 leukemia cells (20) were a kind gift from the laboratory of Dr. R. Herberman, National Cancer Institute. V. I. L. leukemia cells were obtained in our laboratory from the enlarged thymuses of adult rats infected neonatally with the rat-adapted Gross leukemia virus (4).

Results

Description of the Culture System

Feeder layer. The ability of feeder layers prepared from mouse and rat bone marrow, spleen, and thymus to support the in vitro growth of TdT^+ cells from rat and mouse bone marrow was tested. Only a xenogeneic culture system consisting of a mouse bone marrow feeder layer and a rat bone marrow cell suspension was able to consistently generate TdT^+ cells. The converse xenogeneic combination and the syngeneic combinations (rat-rat; mouse-mouse) were ineffective. >99% of the TdT^+ cells that appeared in the mouse-rat cultures were of rat origin, as determined by double immunofluorescence for TdT and the appropriate rat and mouse major histocompatibility antigen (RT-1 and H-2, respectively).

Bone marrow cells from 3–5-wk-old male C57BL/6J, C57BL/10, A/J, A(Thy-1.1), DBA/2J, NZB, NZW, and B6A F_1 strain mice were equally suitable as feeder layers for bone marrow cell suspensions from LEW, DA, LDA F_1, F344, M520, BUF, NIH-r*nu* (*nu/nu, nu/+*), ALB, and NBR strain rats. To standardize the system, bone marrow feeder layers from B6A F_1 mice and bone marrow cell

suspensions from LDA F_1 rats were used in the subsequent experiments, except as indicated.

Serum supplement. To optimally generate TdT^+ cells in vitro it was necessary to supplement the tissue culture medium with 20–30% FBS. However, it was noted that some lots of FBS were more supportive than others.

Inasmuch as TdT^+ cells are sensitive in vivo to the administration of adrenal glucocorticosteroid hormones and to PGE_1 (21, 22),[2] we suspected that the presence of elevated levels of these (and possibly other) steroid hormones in some lots of FBS might inhibit the proliferation of TdT-positive cells in the culture system. To test this, several lots of FBS that only nominally supported the growth of TdT^+ cells in vitro were extracted with activated charcoal (ChXFBS), which has been shown to selectively remove steroid hormones (9, 23). After charcoal extraction, all lots of FBS strongly supported the growth of TdT^+ cells in vitro.

One example of this effect is shown in Fig. 1. In cultures supplemented with 30% unextracted FBS, the number of TdT^+ cells on day 10 was significantly less than that on day 0 (Fig. 1*A*). However, in cultures supplemented with 30% ChXFBS there was only a transient decline in TdT^+ cells, which was followed by an exponential increase, so that the total number of TdT^+ cells was approximately eightfold greater than starting levels by day 10 and 20-fold by day 14 (accounting for 30 and 75% of total nucleated cells, respectively) (Fig. 1*B*). Similarly en-

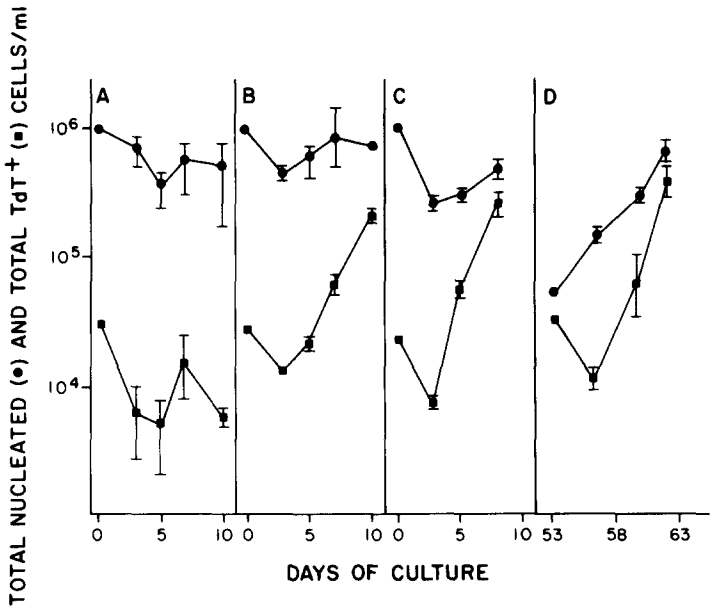

FIGURE 1. Selective growth of LDA F_1 rat TdT^+ bone marrow cells in vitro on B_6A F_1 mouse bone marrow feeder layers in medium supplemented with 30% (*A*) undefined FBS, (*B*) charcoal-extracted FBS, and (*C*) defined Hyclone FBS. Each point represents the mean ± SD of triplicate wells. (●) Total number of nucleated cells/ml of culture (each well contained 2 ml); (■) absolute number of rat TdT^+ cells/ml of culture. (*D*) Kinetics of generation of TdT^+ cells in long-term culture (also see Fig. 3). Note that only 5×10^4 total nucleated cells/ml were inoculated into the cultures described in *D*.

hanced growth of TdT^+ cells was obtained with 30% defined HyClone FBS (Fig. 1*C*), in which the concentration of total hydrocortisone (1.3 ng/ml) was ~60 times below that reported for normal rat plasma (24) and undefined FBS (25).

Reconstitution experiments demonstrated that, among a variety of steroid hormones, only hydrocortisone was able to suppress the generation of TdT^+ cells when added to the culture medium in concentrations at or below physiological levels (calculated as free cortisol, i.e., ~15% of total cortisol). Thus, cultures to which 1 ng/ml hydrocortisone was added on days 0, 2, 4, and 6 contained ~10 times fewer TdT^+ cells on day 7 than did untreated control cultures; and cultures that received 10 ng/ml hydrocortisone contained no detectable TdT^+ cells. As shown in Fig. 2*B*, dexamethasone had similar suppressive effects when used at 10-fold lower concentrations. PGE_1 also inhibited the generation of TdT^+ cells in vitro (Fig. 2*C*), but only when used at concentrations (500 ng/ml) ~250 times greater than that present in the defined FBS (diluted 1:5 in medium). Aldosterone, testosterone, progesterone, and estradiol did not inhibit the generation of TdT^+ cells, even when used at concentrations approximately 1×10^3 to 5×10^4 times above those present in the defined FBS (Fig. 2*D*).

Kinetics of generation of TdT^+ cells in vitro. The kinetics of generation of TdT^+ cells in the primary cultures was linear with respect to time between days 3 and 14, after which the feeder layer began to deteriorate and the number of TdT^+ cells decreased (Fig. 1, *B* and *C*). However, the generation of TdT^+ cells could be maintained for more than 6 mo by serial passage of the nonadherent cells in the culture to fresh feeder layers at 10-d intervals (Fig. 3*A*). Moreover, the

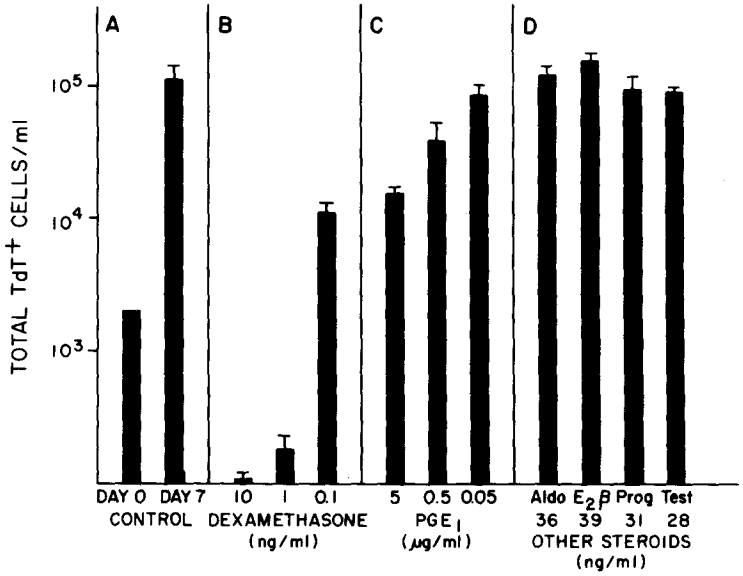

FIGURE 2. Effect of steroid hormones on the growth of rat TdT^+ cells in vitro as compared to (*A*) untreated control cultures. The hormones were administered in the indicated doses on days 0, 2, 4, and 6. The cultures were harvested on day 7. (*B*) Dexamethasone; (*C*) prostaglandin E_1 (PGE_1); (*D*) estadiol 17β ($E_2\beta$), aldosterone (*aldo*), progesterone (*prog*), and testosterone (*test*).

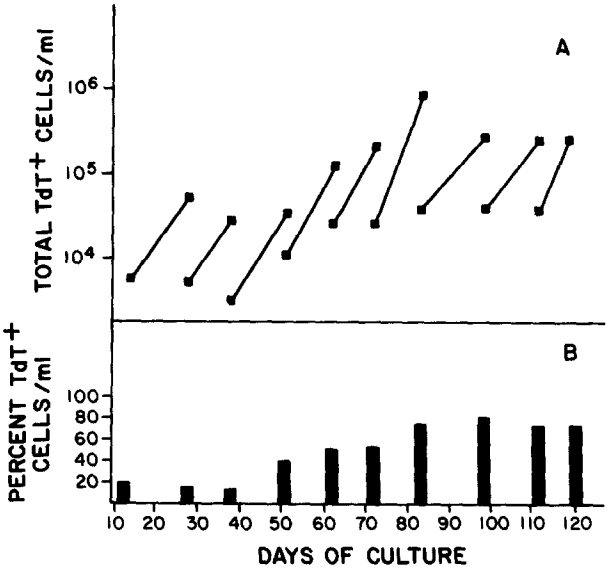

FIGURE 3. Long-term growth of LDA F_1 rat TdT^+ bone marrow cells in vitro maintained by serial passage at 10-d intervals of nonadherent cells to fresh mouse bone marrow cell feeder layers in RPMI 1640 plus 30% defined FBS. (*A*) total TdT^+ cells/ml generated after each transfer between days 10 and 120. (*B*) TdT^+ cells on days of transfer, expressed as the percentage of total nucleated cells/ml of culture medium. The growth of the TdT^+ cells in this culture has now been maintained for >10 mo.

percentage of TdT^+ cells progressively increased with time in culture, reaching a plateau of 75–80% by day 100 (Fig. 3*B*).

The mean doubling time for the TdT^+ cells in primary culture (between day 3 and 10) was 35.0 ± 9.58 h. The apparent mean doubling in the long-term cultures was 71.64 ± 33.95 h (Fig. 3*A*). However, closer inspection of the long-term cultures revealed a lag period of 2–3 d after each transfer (Fig. 1*D*). If this lag is taken into account, the mean doubling time of TdT^+ cells in the long-term cultures was 46.1 ± 12.9 h. ~20% of the TdT^+ cells in both the primary and long-term cultures incorporated [^{3}H]thymidine during a 60-min pulse.

The number of TdT^+ cells that were generated in the primary cultures was directly proportional to the number of rat bone marrow cells that were initially added. Results in Fig. 4 show that the coefficient of linearity (r^2) was 0.97 between doses of 10^3 and 10^6 bone marrow cells. In long-term cultures there is a similar dose-response relationship between the number of nonadherent cells transferred and the number of TdT^+ cells generated at a given time point. However, a plateau was reached in these cultures when approximately 5×10^5 to 1×10^6 TdT^+ cells were generated (data not shown). Refeeding the cultures during the plateau phase did not cause a renewed increase in the growth of TdT^+ cells, whereas splitting and refeeding the cultures did.

Fate of CFU-S and CFU-C. As indicated in Fig. 1*B*, there was a progressive decrease in the number of TdT^- cells in the cultures with time. By day 7, almost all of the nonlymphoid cells in the suspension were macrophages. Of these, 95% stained for mouse, but not rat, histocompatibility antigens, indicating that they

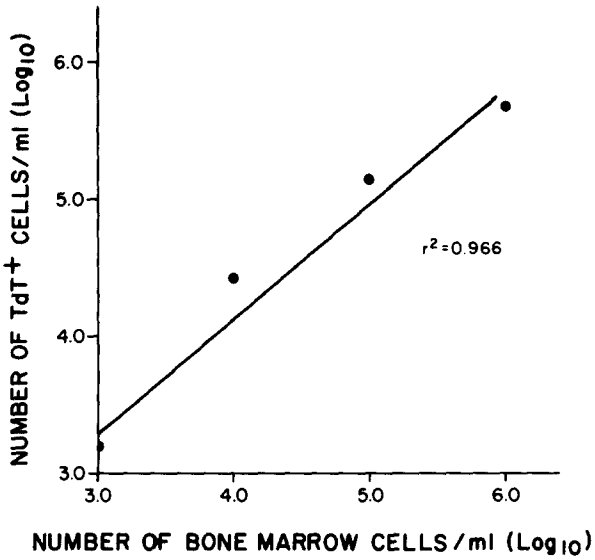

FIGURE 4. Dose-response relationship between the number of nucleated rat bone marrow cells added to the cultures at day 0 and the number of rat TdT^+ cells present at day 10.

TABLE I

*Presence of Rat CFU-S and GM-CFC in Cultures of Various Ages**

Days of culture	CFU-S/10^6 cells	GM-CFC/10^5 cells
0	15.6 ± 4.0	87.9 ± 8.8
1	6.4 ± 1.5	ND
3	5.9 ± 0.7	ND
4	4.9 ± 1.6	ND
6	0.3 ± 0.5	ND
10	0.4 ± 1.3	31.7 ± 4.8
148	0	ND
195	ND	0
316	ND	0

* Results are expressed as the mean ± standard deviation of the number of in vivo (CFU-S) and in vitro (GM-CFC) colonies. Values of 0 indicate that no colonies were detected above control levels. ND, not determined.

were derived from the feeder layer. Similarly, no rat origin myeloid or eythroid cells were identified in the long-term cultures.

To determine whether CFU-S and GM-CFC survived or proliferated in these cultures, the nonadherent cells were harvested at timed intervals and tested in the appropriate in vivo and in vitro assays. The results in Table I show that there was a marked decrease in CFU-S and GM-CFC (presumably of rat origin) by day 10; and that neither CFU-S nor GM-CFC were detectable in the long-term cultures.

Conditioned medium (CM) from primary and long-term cultures of rat TdT^+ cells was tested alone or mixed with SCM in the GM-CFC assay. Neither stimulatory nor inhibitory activity was identified in the TdT-CM (data not shown).

Factors stimulating TdT^+ cell growth. Bovine pituitary fibroblast growth factor (FGF), guinea pig BMX (GP-BMX), and rat BMX (R-BMX) have been reported to stimulate the growth of cells of mesodermal origin, including hemopoietic cells (26, 27).

When 1 ng/ml FGF was added to the medium on days 0, 2, 4, and 6, a twofold increase in the number of TdT^+ cells above levels in control cultures was observed on day 7. 10 ng/ml FGF produced a fourfold increase and 100 ng/ml FGF produced a 10-fold increase in the number of TdT^+ cells.

A threefold increase in the number of TdT^+ cells in 7-d cultures was observed when 1 μg/ml GP-BMX was added to the medium on days 0, 2, 4, and 6. A fourfold amplification was induced by 10 μg/ml GP-BMX. Similar results were obtained with R-BMX (data not presented). However, mouse BMX (M-BMX), which has not been found to have mesodermalizing activity (26), had no significant effect on the growth of TdT^+ cells in vitro.

A mixture of 10 ng/ml FGF and 1 μg/ml GP-BMX caused a nearly 10-fold increase in the number of TdT^+ cells above levels in control cultures.

In addition to the preceding mesodermalizing factors, which required 30% ChXFBS to be maximally effective, a series of eight factors commonly found to reduce the serum requirements of cells in vitro (9) were tested (transferrin, 10 μg/ml; nerve growth factor, 30 ng/ml; triiodothyronine, 1×10^{-11} M; endothelial cell growth factor, 50 μg/ml; gimmel factor, 10 μg/ml; selenium, 1×10^{-9} M; LiCl, 1×10^{-9} M; and Fe SO_4, 1×10^{-10} M). Of these, a combination of triiodothyronine (T_3), transferrin, and selenium permitted the concentration of ChXFBS to be reduced to 5%. However, the addition of these three factors to cultures containing 30% ChXFBS did not further significantly increase the generation of TdT^+ cells.

Properties of the TdT^+ Cells Generated In Vitro

The properties of the TdT^+ cells that were generated in vitro were compared with those of their in vivo counterparts in normal rat bone marrow and thymus. Without exception, the cultured TdT^+ cells resembled normal TdT^+ bone marrow cells, but not thymocytes.

Morphology. The immunofluorescence pattern of TdT staining and the mormphological appearance of the TdT^+ cells generated in vitro are shown in Figs. 5 and 6. Like normal TdT^+ bone marrow cells and unlike most TdT^+ thymocytes (3, 15), the majority of cultured TdT^+ cells were basophilic lymphoblasts that had a diffuse pattern of intranuclear TdT fluorescence. As determined by FACS analysis and sorting (Fig. 7), the cultured TdT^+ cells also had the same size distribution profile as did normal TdT^+ bone marrow cells, which are significantly larger than most TdT^+ thymocytes.

Antigenic phenotype. Bone marrow cells from LDA F_1, M520, and ALB strain rats were used to establish long-term cultures of TdT^+ cells. As shown in Table II, all of the TdT^+ cells from day 10 and day 70 cultures had the same "null" antigenic phenotype as did noncultured TdT^+ bone marrow cells. All were strongly positive for both the RT-1 rat major histocompatibility antigen and the Thy-1.1 alloantigen, the latter being expressed on immature bone marrow cells of all lineages in the rat (17). ~30% of the day 10 TdT^+ cells and 80% of the

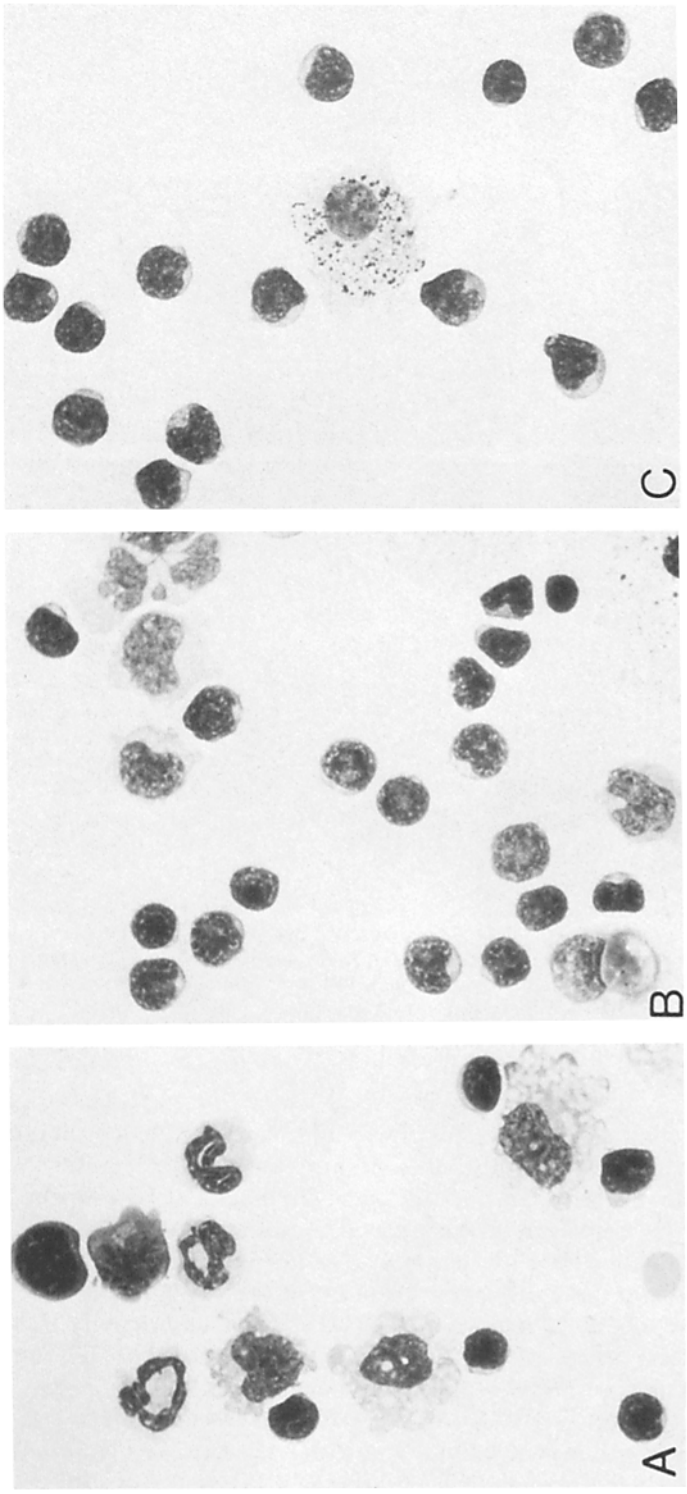

FIGURE 5. Morphology of the rat bone marrow cells present in (*A*) day 3, (*B*) day 10, and (*C*) day 50 cultures. Note the progressive shift to lymphoid populations with time in vitro. The percentage of TdT^+ cells in these cultures was (*A*) 1.9 ± 0.52, (*B*) 29.6 ± 4.1, and (*C*) 46.4 ± 12.9. Wright-Giemsa stain. × 1,000.

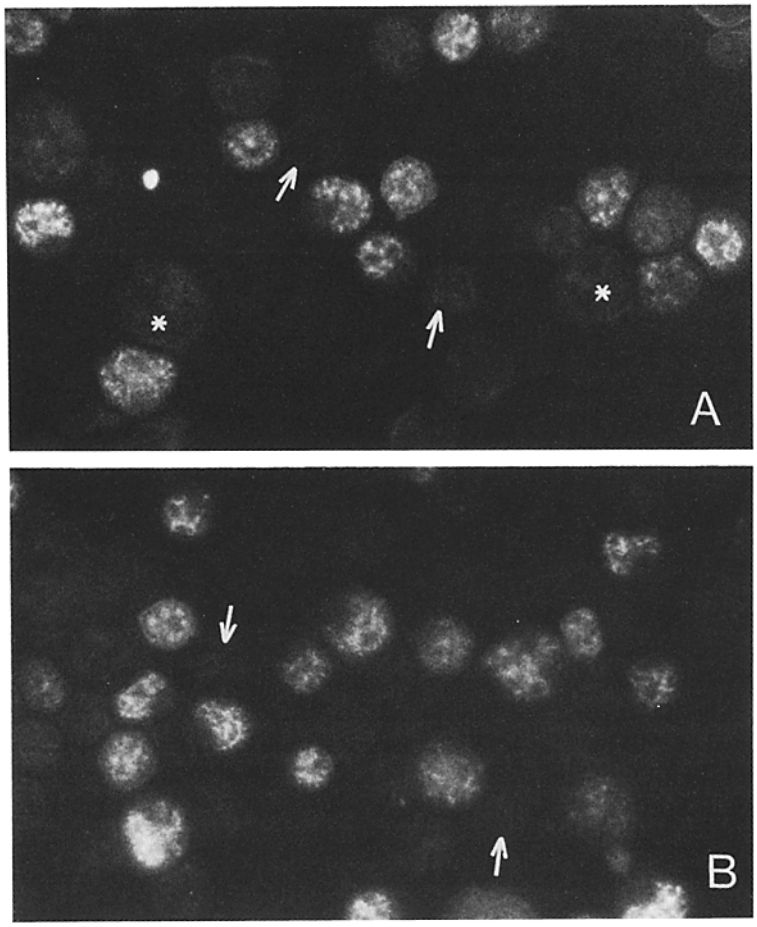

FIGURE 6. Rat bone marrow cells in day-8 cultures stained for TdT by indirect immunofluorescence. ~35% of the total nucleated cells in these samples were TdT^+, as indicated by bright intranuclear fluorescence. These were medium-size lymphoblasts. Many smaller lymphoid cells were TdT^-. These appear as nonfluorescing "ghosts" in the photomicrograph (arrows). Scattered monocytes and macrophages were also present (asterisks). See Fig. 5*B* for the different morphological cell types present.

day 70 TdT^+ cells were positive for the W3/13 antigen, which, in addition to being a pan–T cell antigen, is also present on many developing cells in rat bone marrow (11). However, in contrast to TdT^+ thymocytes, none of the cultured TdT-positive cells displayed T cell–specific differentiation antigens; and in contrast to developing B cells, none had detectable surface or cytoplasmic immunoglobulin and none displayed Ia antigens.

Properties of the TdT. As in vivo, the TdT enzymatic activity in vitro was proportional to the percentage of TdT^+ cells present and the TdT was a single peptide chain of mol wt 58,000 (6, 19, 21).

Leukemogenic Potential. 1×10^5 or 1×10^6 TdT^+ cells from day 10 and day 50 cultures of either Lewis or LDA F_1 rat bone marrow were injected intravenously into sublethally irradiated (675 rad) LDA F_1 recipients. Other recipient

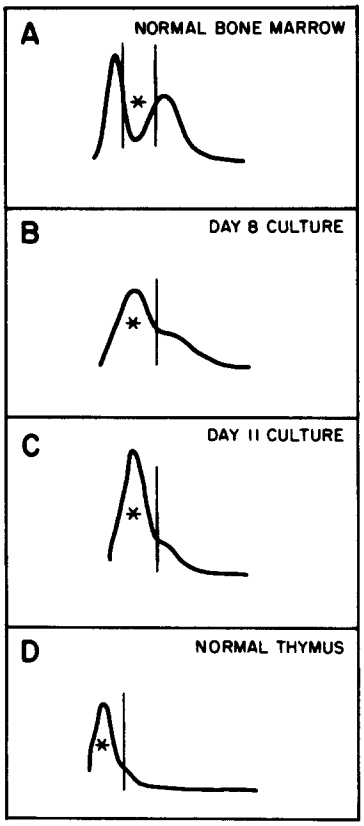

FIGURE 7. Forward angle light scatter profile determined by the FACS of (*A*) normal rat bone marrow; (*B*) day-8 cultured bone marrow cells, (*C*) day-11 cultured bone marrow cells, and (*D*) normal rat thymocytes. 80% or more of the TdT^+ cells were located between the cursors in *A* and to the left of the cursors in *B–D* (see asterisks). The modal size distribution of the TdT^+ cells in *B* and *C*, as determined by sorting on the FACS, is identical to that in *A* and significantly larger than that in *D*.

rats were injected with allogeneic (G-1) or syngeneic (V. I. L.) TdT^+ rat leukemia cells or with media alone. As few as 1×10^4 G-1 cells killed the recipient rats within 4 wk, and 1×10^6 G-1 or V. I. L. leukemic cells killed the recipients within 16 d (data not shown). At autopsy, all of the animals receiving either G-1 or V. I. L. cells had massive leukemic infiltrates in their tissues. In contrast, none of the recipients of the cultured TdT^+ cells or media alone displayed gross or histologic evidence of leukemia when autopsied 8 wk after cell transfer.

Discussion

The present culture system is one of only two long-term systems that have been reported to selectively generate primitive lymphoid cells in the absence of detectable CFU-S. Whitlock and Witte (28) and Kurland et al. (29) have described a system that preferentially generates mouse pre-B cells in vitro. Our culture system preferentially generates undifferentiated rat TdT^+ cells, which we postulate are early members of the T cell lineage (see below). Both of these systems

TABLE II
*Antigenic Phenotype of Rat TdT$^+$ Cells**

Antigens‡	In vitro TdT$^+$ cells§	Normal bone marrow TdT$^+$ cells	Thymic TdT$^+$ cells
RT-1	++	++	++
Thy 1.1	++	++	++
W3/13	+ (++)	+	++
OX-19	−	−	++
W3/25	−	−	++
OX-8	−	−	++
OX-3,4	−	−	−
RT-7^a (A.R.T. 1^a)	−	−	++
RT-6^a (A.R.T. 2^a)	−	−	−
A.R.T. 3‖	−	−	+
cIgM	−	−	−
sIgM	−	−	−

* Determined by double immunofluorescence for intranuclear TdT and surface antigenic markers. (++) 76–100% of TdT$^+$ cells are positive; (+) 1–75% of TdT$^+$ cells are positive; (−) <1% of TdT$^+$ cells are positive.
‡ See Materials and Methods and Results for description.
§ Cells analyzed from day 10 and day 70 cultures. Comparable results were obtained, excepting W3/13, in which approximately 30 and 80% of TdT$^+$ cells were positive in day 10 and day 70 cultures, respectively.
‖ Provisional designation.

are modifications of the Dexter long-term culture system (30), which selectively maintains CFU-S and GM-CFC and contains only low levels of presumptive lymphoid precursor cells (8, 31, 32). In contrast to these long-term culture systems, Reimann and Miller (33) have described a short-term, limiting dilution, microculture system that apparently permits the growth and differentiation of cytolytic T cells from Thy-1$^-$ precursors from nude mouse bone marrow. Several other culture systems have been described that generate colonies of differentiated T or B cells in semi-solid medium (34, 35). However, these cultures appear to preferentially detect the progeny of relatively mature lymphocytes.

A number of common features are shared by our lymphoid culture system and that of Whitlock and Witte (28), including: (*a*) the use of FBS as the serum supplement; (*b*) the need for reduced levels of adrenal glucocorticosteroid hormones; (*c*) the presence of adherent monolayers of dispersed mouse bone marrow cells; and (*d*) incubation at 37°C. In contrast, the standard Dexter culture system uses normal horse serum as the serum supplement, hydrocortisone frequently is added, the three-dimensional orientation of the bone marrow stroma is maintained in the feeder layer, and the cultures are incubated at 33°C (31, 36).

These differences presumably account not only for the maintenance of lymphoid precursor cells in our culture system, but for the inability of these cultures to support the growth of CFU-S and GM-CFC. For example, adrenal corticosteroid hormones have been shown to have profound lymphocytolytic effects on TdT$^+$ bone marrow cells and thymocytes and on immature B lymphocytes (17, 21, 23, 37, 38), but to improve myelopoiesis and prolong the survival of CFU-S

in vitro (28, 29, 36; and unpublished results). Hence subphysiological concentrations of adrenal corticosteroids would be expected to favor the growth of early lymphoid cells as opposed to CFU-S and myeloid progenitor cells. Similarly, we have shown that pharmacological doses of PGE_1 inhibit the production of TdT^+ bone marrow cells and thymocytes in vivo (22);[2] and that a concentration of PGE_1 (500 ng/ml) roughly equivalent to that normally present in the thymus microenvironment (39) inhibits the generation of TdT^+ cells in vitro. Therefore, it is possible that the relative absence of myelopoiesis in our culture system, and the presumed decrease in monocyte-derived PGE_1 (38), might further favor the outgrowth of TdT^+ cells. Although this remains to be tested, it may help to explain why in standard Dexter cultures, which favor myelopoiesis, TdT^+ bone marrow cells are not generated at levels greater than those normally seen in vivo (~2%) (8). That the reciprocal generation of lymphoid cells and myeloid cells is not restricted to the in vitro situation has recently been illustrated by Engelhard et al. (40) in cases of cyclic neutropenia. Thus, our results suggest that both adrenal corticosteroids and PGE_1 may be involved in the complex feedback loops that normally regulate the relative production of lymphoid and myeloid cells during hemopoiesis.

A second requirement for the growth of rat bone marrow TdT^+ cells in our culture system is the presence of a mouse bone marrow feeder layer. Neither syngeneic, allogeneic, nor other xenogeneic feeder layers from bone marrow or other tissues could be substituted. Moreover, the mouse bone marrow feeder layer could not be replaced by CM from the feeder layers (unpublished). Therefore, it seems that the growth of TdT^+ cells is not caused simply by rat bone marrow lymphocytes reacting against xenogeneic antigens or solely by factors released by the feeder layers. Rather, the results of ongoing studies suggest that the physical adherence of even more primitive rat lymphoid precursor cells (pre-TdT^+ cells) to the feeder layer is necessary for the generation of the nonadherent TdT^+ cells.[3] For the moment, there is no obvious explanation as to why mouse bone marrow feeder layers do not also support the growth of mouse TdT^+ cells or, conversely, why rat feeder layers do not support the growth of rat TdT^+ cells. However, it has been noted that the adherent cells grown from rat bone marrow significantly differ morphologically from those grown from mouse bone marrow (unpublished results).

By serially passaging the nonadherent cells onto fresh feeder layers, we have managed to generate TdT^+ cells in vitro for >10 mos. These cultured cells appear to be indistinguishable according to morphology, size, antigenic phenotype, and cortisone sensitivity from their normal, undifferentiated counterparts in bone marrow. They are generated with equal facility from the bone marrow of normal and congenitally athymic rats. Moreover, they do not cause leukemia after injection into irradiated, histocompatible recipients.

It might be argued that the persistence of TdT^+ cells in our culture system is due to the selection of one or several lines of rapidly proliferating TdT^+ cells. This seems unlikely for several reasons. First, there is a strict dose-response

[3] Medlock, E. S., D. L. Greiner, J. Hayashi, and I. Goldschneider. A selective culture system for generating terminal deoxynucleotidyl transferase-positive (TdT^+) lymphoid precursor cells in vitro. II. Properties of the lymphopoietic stem cells. In preparation.

relationship between the number of bone marrow cells inoculated and the number of TdT^+ cells generated in the primary cultures. Second, there is only a short lag phase between the time of initiation of the cultures and the logarithmic generation of TdT^+ cells. This is particularly true when the bone marrow cell inoculum is obtained from dexamethasone-treated donors, in which case no lag phase whatsoever is observed.[3] Third, the mean doubling time for the generation of TdT^+ cells is similar in both short-term and long-term cultures, indicating that no particular clone had a proliferative advantage over any other.

Our working hypothesis, based on previously published evidence (1, 2, 16) is that most of the TdT^+ cells in our culture system are early members of the T cell lineage. Three additional pieces of evidence further support this assumption. First, we have not detected cytoplasmic Ig (or surface Ig) in any of the rat TdT^+ cells generated in vitro. Second, the cultured TdT^+ cells appear to bear a T-100 antigen (41) that is present on thymocytes and peripheral T cells but not on mature or immature B cells (unpublished). Third, bone marrow cells from 4-wk-old rats infected neonatally with the rat-adapted Gross leukemia virus (4) selectively generate TdT^+ leukemias of the helper/inducer T cell phenotype ($OX\text{-}8^-$, $W3/25^+$) in our culture system.[4] Nonetheless, the possibility has not been excluded that some of the cultured rat TdT^+ cells are primitive members of the B cell lineage. Such a possibility has been suggested by Whitlock and Witte (28), who found that 7 of 11 clones of Abelson virus–transformed mouse pre-B cells in their culture system expressed TdT. However, closer examination of this system has revealed that only 1 of 6 transformed pre-B cell lines was TdT^+ when the nontransformed precursors were cloned before infection with the Abelson virus (42). Moreover, the TdT^+ clone appears to have originated from a precursor whose μ heavy chain gene had not been rearranged.

In vivo adoptive transfer studies and in vitro induction studies, currently in progress, should help to clarify the lineage relationships of the cultured TdT^+ cells in our system. Whatever their developmental potential may prove to be, the ability to selectively generate TdT^+ cells in vitro offers a heretofore unavailable opportunity to study the very early stages of normal, as well as abnormal, lymphopoiesis. One unique advantage of this culture system is that it permits the cellular interactions and humoral factors that influence the proliferation of primitive lymphoid cells to be distinguished from those that influence their differentiation. Another advantage of this culture system is that it offers the opportunity to conduct the critical cloning experiments needed for studies of the generation of the receptor repertoire and the induction of self-tolerance. While the present system will only permit such analyses to be done on rat lymphopoietic cells, results of preliminary experiments suggest that it may be possible to adapt this xenogeneic culture system to generate mouse and, ultimately, human bone marrow TdT^+ cells in vitro, thereby further increasing its potential utility.

[4] Medlock, E. S., D. L. Greiner, R. W. Barton, and I. Goldschneider. A selective culture system for generating terminal deoxynucleotidyl transferase-positive (TdT^+) lymphoid precursor cells in vitro. III. Generation of preleukemic and leukemic TdT^+ cells after infection with the rat-adapted Gross leukemia virus. In preparation.

Summary

A primary xenogeneic culture system has been devised that selectively generates undifferentiated TdT^+ lymphoblasts from rat bone marrow under conditions that do not support the growth or maintenance of rat colony-forming unit–spleen (CFU-S) or granulocyte/macrophage colony-forming cells (GM-CFC). The culture system requires a mouse bone marrow feeder layer, and a serum supplement that has markedly reduced levels of cortisol. The growth of TdT^+ cells can be significantly enhanced by the addition of mesodermalizing factors (*e.g.*, fibroblast growth factor, guinea pig bone marrow extract) to the culture medium, and the serum supplement can be decreased by the addition of selenium, transferrin, and T_3. The cultured TdT^+ cells are antigenically "null" cells that further resemble their normal counterparts in bone marrow with respect to morphology, size, cortisone sensitivity, and pattern of TdT fluorescence. The TdT^+ cells are generated with equal facility from bone marrow of normal and congenitally athymic rats, can be maintained in logarithmic growth for at least 10 mos by serial passage in vitro, and do not cause leukemia when infused into irradiated recipients. Although the lineage relationships of these immature lymphoid cells have not yet been established, our working hypothesis, based on preliminary evidence, is that the cultured TdT^+ cells are primitive members of the T cell series.

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