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Human Bone Marrow and Peripheral Blood T Lymphocyte Depletion: Efficacy and Effects of Both T Cells and Monocytes on Growth of Hematopoietic Progenitors

By Lee Levitt, Thomas J. Kipps, Edward G. Engleman, and Peter L. Greenberg

The efficacy of four separate methods of human bone marrow T lymphocyte depletion was assessed, and the effect of T cells and monocytes on in vitro growth of marrow (CFU-GEMM, BFU-E, and CFU-GM) and peripheral blood (BFU-E) hematopoietic progenitors was determined. Extent of T cell depletion was assessed by multiparameter fluorescent cell sorter (FACS) analysis and by functional studies. Cells staining positively by FACS analysis for one or more of three separate fluorescent pan-T cell monoclonal antibodies (MCABs) comprised 8.4% to 9.5% of control marrow mononuclear cells (MNCs). T cells constituted 3.2% to 5.1% of marrow following single, sequential, or combination treatment with two different pan-T cell MCABs (Leu 1 and TM1) plus complement, 1.5% to 2.2% of marrow following solid-phase immunoabsorption ("panning"), 0.2% of marrow after sheep cell rosetting, and only 0.05% of marrow after FACS selective cell sorting and gated separation. T cells made up 59% to 73% of control peripheral blood MNCs and 0.8% to 2.8% of peripheral MNCs following sheep cell rosetting plus treatment with Leu 1 MCAB and complement. Mitogen (PHA, Con A) and allogeneic MLC-induced blastogenic responses (stimulation indices, experimental/control or E/C) revealed a concordant decrement in marrow T cell function after MCAB plus complement (E/C of 3.9 to 9.0), after panning (E/C of 1.6 to 3.5) and after sheep cell rosetting (E/C of 0.7 to 1.3), compared with control marrow (E/C of 5.3 to 15.7). After T cell depletion, marrow BFU-E growth was 95% to 120% of control, CFU-GM growth was 90% to 108% of control, and

CFU-GEMM growth was 89% to 111% of control. Marrow T cell and/or monocyte depletion did not alter erythropoietin-dependent BFU-E growth in the absence of Mo-conditioned medium (81% to 95% of control), and the addition of as many as 50 to 100×10^3 purified marrow monocytes or T cells to 10^5 autologous nonadherent T cell-depleted marrow target cells had a negligible ($P > .1$) effect on marrow BFU-E growth in vitro. Peripheral blood (PB) BFU-E/ 10^5 T-depleted target cells were $106\% \pm 19\%$ of expected; PB BFU-E growth was significantly diminished after monocyte depletion alone ($7\% \pm 6\%$ of expected) or after monocyte plus T cell depletion ($8\% \pm 4\%$ of expected). Addition of as many as 150×10^3 T cells to 10^5 autologous nonadherent T cell-depleted PB "null" cells produced only small (18% to 30% of expected) increments in BFU-E growth, whereas addition of autologous monocytes to "null" target cells restored PB BFU-E growth to 82% to 94% of expected. We conclude that (1) T lymphocyte depletion does not appreciably alter in vitro growth of human marrow (BFU-E and CFU-GM) or PB (BFU-E) hematopoietic progenitors; (2) that monocytes are a major source of burst-promoting activity for PB but not marrow BFU-E erythropoietin-dependent differentiation; and (3) that functional and immunologic marrow T-cell depletion is more effectively achieved with panning, sheep cell rosetting, or gated FACS separation, rather than by treatment with pan-T cell (Leu 1, TM1) MCAB plus complement.
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GRAFT-V-HOST disease (GVHD) is a source of considerable morbidity and mortality in human allogeneic bone marrow transplantations.^{1,2} Despite the use of sibling donors matched at the major histocompatibility complex and despite postgrafting immunosuppression, GVHD occurs in approximately 50% of patients with successful marrow engraftment^{1,3,4} and is a major contributor to the impaired immunologic reactivity, debility, and compromised mucosal barriers seen in marrow-graft recipients. Patients with moderate to severe (grades II to IV) GVHD have an overall fatality rate of 40% to 50%.³ Survival falls markedly for marrow transplant recipients greater than 30 years of age, owing almost entirely to an increased frequency and severity of GVHD in older patients.⁴ Donor-recipient HLA identity is a usual prerequisite for marrow transplantation, and the generally severe degree of GVHD accompanying infusion of histoincompatible cells has also markedly restricted the pool of marrow donors available for any given recipient.¹

Experimental studies indicate that the cell responsible for initiating the graft-v-host reaction is a

post-thymic (immunologically mature) donor T lymphocyte.⁵⁻⁸ Recent work in rodents,⁵ canines,⁹ and primates¹⁰ has demonstrated that marrow depleted of alloreactive T lymphocytes can be used to reconstitute lethally irradiated recipients across major histocompatibility barriers without producing severe GVHD. A variety of techniques have been used in animal models for the efficient in vitro eradication of T lymphocytes from donor marrow, including discontinuous albumin

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gradient centrifugation,¹¹ continuous in vitro marrow culture,¹² T cell opsonization or complement-mediated cytolysis using either heteroantisera¹³ or monoclonal antibodies,⁵ soybean and peanut agglutinin fractionation,¹⁴ and chemoseparation with such reagents as 4-hydroperoxycyclophosphamide and mitomycin.¹⁵ The optimal method for human marrow T cell depletion, however, remains to be determined. Efficacy of marrow T cell depletion has not always been carefully assessed, and the effect of T lymphocyte depletion on the growth of multiple human hematopoietic progenitors remains controversial. Careful assessment of the adequacy of marrow T cell depletion becomes a particularly important issue in lieu of recent animal⁶ and human¹⁶ studies, indicating that very small numbers of residual donor marrow T lymphocytes can apparently still mediate GVHD, particularly following histoincompatible marrow transplantation.

The present studies were designed to assess the efficacy of marrow T cell depletion by solid-phase immunoabsorption and by multiparameter fluorescent cell sorter (FACS)-gated separation, and to compare these results with those obtained using standardized sheep cell rosette fractionation and monoclonal antibody-dependent complement-mediated lysis. Extent of T cell depletion was carefully assessed by both functional studies and cell sorter analysis. These studies have enabled us to further delineate the role of accessory cells in the regulation of hematopoiesis,¹⁷⁻²⁵ and to clarify the role of T lymphocytes and monocytes in the in vitro proliferation and differentiation of human marrow and peripheral blood hematopoietic progenitors.

MATERIALS AND METHODS

Marrow Samples

All studies were approved by the Stanford University Human Experimentation Committee. Two to four milliliters of marrow aspirate or 20 to 40 mL of whole blood from normal adult donors were drawn into heparinized syringes, layered over Ficoll-Hypaque gradients ($d = 1.077$ g/mL), centrifuged, and interface buoyant mononuclear cells (MNCs) collected and washed three times in Iscove's modified Dulbecco's medium (IMDM). Marrow or peripheral blood nonadherent MNCs were obtained after two consecutive 90-minute adherence procedures at 37 °C in polystyrene dishes with 7.5% fetal calf serum (FCS) at 10^7 cells per milliliter.^{22,26} Nonadherent cell suspensions were sometimes further depleted of monocytes by panning with Leu M3 monoclonal antibody (MCAb). Nonadherent buoyant (NAB) mononuclear cells obtained in this manner were always <2% monocytes by Wright-Giemsa and esterase staining and immunofluorescence labeling with Leu M3 and 61D3 MCAs.^{27,28} Adherent cells (>95% monocytes by esterase and Leu M3 immunofluorescence) were obtained from peripheral blood and marrow by a slight modification of the technique of Shaw²⁹ after incubation of MNCs with 10% AB serum in tissue culture dishes (coated overnight at 4 °C with FCS) for 90 minutes at 37 °C. In control

experiments, cultures of monocytes alone never yielded >2 to 3 BFU-E/ 10^5 cells plated.

Assay Systems for Hematopoietic Progenitors

Marrow or peripheral blood MNCs were cultured (5 to 15×10^4 /mL) with 1 unit human urinary erythropoietin (NIH, 1,140 U/mg protein) in triplicate 1-mL aliquots containing 1.1% methylcellulose, 50 μ mol/L 2-mercaptoethanol, and 30% FCS, with or without 1% Mo-conditioned medium (Mo CM, a medium conditioned by a T lymphoblast cell line, containing both myeloid colony-stimulating activity and erythroid burst-promoting activity [BPA] and kindly provided by Dr David Golde³⁰).³¹ Cells were sometimes cultured in 5% to 20% FCS and 1% bovine serum albumin to facilitate detection of BPA. Cultures were incubated at 37 °C in 5% CO₂, and colonies were enumerated on days 12 to 14 to assess committed erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitors, and multipotential (CFU-GEMM) hematopoietic stem cells. Marrow mature erythroid (CFU-E) progenitors were scored on days 5 to 7 and large (> eight to 16 clusters) BFU-E were scored after 18 to 21 days of incubation.²⁴ The multilineage composition of CFU-GEMM was confirmed by micropipet selection and Wright-Giemsa staining of representative colonies from each experiment. Marrow CFU-GM were also independently assessed in cultures containing 15% human placental-conditioned medium, in place of Mo CM, as a source of myeloid colony-stimulating activity,³² and colonies were enumerated after seven to ten days' incubation. Peripheral blood MNCs depleted of T lymphocytes were obtained by neuraminidase-treated sheep erythrocyte rosette fractionation; nonrosetting cells were subsequently treated with Leu 1 pan-T cell monoclonal antibody (MCAb) plus rabbit complement (see later). Peripheral blood MNCs were plated at low concentrations (3 to 10×10^4 /mL) to further reduce target cell contamination with the negligible numbers of T lymphocytes remaining after T cell depletion. Cultures of peripheral blood T cells alone never contained >2 BFU-E/ 10^5 cells plated. Fractionated MNCs were resuspended to concentration after posttreatment hemacytometer counts and the expected number of BFU-E per 10^5 T cell-depleted target cells was calculated by the formula:

(BFU-E/ 10^5 control MNC)

$$\times \left(\frac{100}{100 - (\% \text{ T cells in control MNCs} - \% \text{ T cells in T-depleted MNCs})} \right) = \text{expected BFU-E.}$$

Analogous calculations were performed to assess expected numbers of BFU-E after either monocyte depletion or both T cell and monocyte depletion from blood and marrow MNCs. Simultaneous cultures of autologous unfractionated MNCs were performed in all depletion and add-back experiments. In parallel experiments, T-depleted peripheral blood target cells were resuspended, prior to plating, to original volumes based on pretreatment cell counts.

In some experiments, enriched populations (0.2 to 10.0×10^4 , ie, 1% to 50%) of either marrow monocytes (>95% positive by esterase stain and Leu M3 immunofluorescence) or marrow T lymphocytes (obtained from the bound MNC fraction of marrow panned with Leu 1 MCAb; >96% T cells by fluorescence microscopy) were added back to cultures containing 1 to 2×10^5 autologous marrow nonadherent MNCs previously depleted of T cells by FACS separation or panning. Control groups included enriched marrow T cells or monocytes, T cell-depleted and unfractionated marrow MNCs; cultures were assayed separately but simultaneously in the presence and absence of Mo CM, for growth of marrow BFU-E. In further experiments, 20 to 150×10^3 enriched peripheral blood T cells obtained after rosette fractionation, or 25 to 200×10^3 blood monocytes obtained by adherence separation, were either cultured

alone or added back to 5 to 10×10^4 autologous nonadherent T cell-depleted peripheral blood MNCs before assessment of observed and expected BFU-E colony growth.

Methods for Marrow and Peripheral Blood T Lymphocyte Depletion

Antibody-dependent complement (C')-mediated cytotoxicity. Two separate nonmitogenic pan-T lymphocyte MCABs directed against separate antigenic determinants were used in these experiments. 17F12/Leu 1 (provided by R. Levy) is a complement-fixing antihuman MCAB that recognizes a 67,000 MW glycoprotein antigen common to virtually all peripheral blood T lymphocytes, most medullary thymocytes, and a variety of T cell lines.³³ TM1 is an IgM complement-fixing MACb (provided by F.C. Grumet) that recognizes a protein on T cells closely associated with the sheep RBC E-rosette receptor; it is strongly cytotoxic for human peripheral blood T lymphocytes and T cell lines at dilutions up to 1:1,000.³⁴ Three separate lots of both Leu 1 and TM1 MCAB were independently assessed for blood and marrow cytotoxicity. MCABs directed against human Ia-like (HLA-DR) antigenic determinants (MCABs 2.06, 1.35, and 1.41, provided by Hugh McDevitt) were also used in some control experiments; these antibodies have been characterized previously with defined specificities against a variety of human B lymphocytes and B cell lines, as well as against alloreactive T lymphocytes and monocyte subsets.³⁵ Suspensions (0.8 to 80.0×10^6 cells/mL) of marrow or blood MNCs in the presence and absence of 0.1% sodium azide were incubated singly or sequentially with varying (1:10 to 1:10⁶ dilutions; 1 to 30 μ g antibody per 10^6 cells) concentrations of MCABs or control fluids for 30 minutes at either 4 °C or at 23 °C, followed by incubation at 23 °C for 2.5 hours with or without pretested rabbit complement (Pel-Freez, Rogers, Ark).³⁶ In an independent assay, 0.6 mL marrow MNCs (0.1 to 10.0×10^7 cells/mL suspended in serum-free medium containing 5% human serum albumin) were mixed with 0.3 mL rabbit C' and 0.02 mL MCAB and incubated at 37 °C for 60 minutes before plating or further analysis. Antiglobulin-enhanced complement-dependent cytotoxicity was performed with 1:10 to 1:50 dilutions of goat anti-mouse immunoglobulin antiserum (provided by S. Ferrone) according to previously published methods.³⁷

Density-gradient fractionation after treatment with sheep RBCs. Suspensions of marrow or peripheral blood MNCs (5×10^6 /mL) were rosetted with neuraminidase-treated sheep erythrocytes (washed fresh red blood cells in a 5% suspension with IMDM), gently pelleted, and then separated by Ficoll-Hypaque density centrifugation. The nonrosetting interface was either analyzed directly or treated with either one of the two pan-T lymphocyte MCABs with or without complement before plating. Peripheral blood MNCs were recovered from the rosetted pellet by treatment with 0.155 mol/L NH_4Cl ; these T cell-enriched fractions were 96% to 100% rosette positive and contained <3% monocytes, based on esterase staining and Leu M3 immunofluorescence microscopy. The rosetted pellet always contained <2 BFU-E per 10^5 cells plated.

Solid-phase immunoabsorption ("panning"). Polystyrene Petri dishes were coated with 100 μ g of affinity-purified goat anti-mouse IgG (Tago Inc.) or goat anti-mouse IgM (Tago, Inc, Burlingame, Calif) diluted in 10 mL of 0.05 mol/L TRIS buffer.³⁸ After a 40-minute incubation at 23 °C, the buffer was decanted and the dishes gently washed five times with cold phosphate-buffered solution (PBS). Marrow cell suspensions (20×10^6 cells in 75 μ L human AB serum) were coated with 50 to 100 μ g of either Leu 1 or TM1 MCAB and incubated at 4 °C for 20 minutes, washed, centrifuged, and then added to the antibody-coated dish (indirect "panning") and incubated for 70 minutes at 4 °C, swirling the dish once midway through the incubation to redistribute unattached cells. Cells bound

to the coated dishes and nonbound cells were separately recovered by differential elution with cold PBS-1% FCS. Nonbound cells were either analyzed directly (FACS analysis, mitogen-induced blastogenic assays, and plating for assessment of hematopoietic progenitors) or first subjected to repeat panning with either Leu 1 or TM1 before assay. In almost all cases, >95% of bound cells and <5% of nonbound cells stained positively with the MCAB used for panning.

Selective cell sorting by FACS-gated separation. Suspensions of either peripheral blood or marrow MNCs were first washed with staining medium (with or without 0.1% sodium azide), and cell aliquots were coated with a combination of two separate pan-T cell (Leu 1 and Leu 5) fluoresceinated MCABs (1 μ g of each antibody per 10^6 cells at a concentration of 25 μ g of antibody per milliliter of staining medium).^{39,40} Control noncoated samples were treated with staining medium alone. After addition of antibody and/or staining medium, cells were incubated for 30 minutes at 4 °C, washed and resuspended to a final concentration of 3 to 5×10^6 cells per milliliter before FACS analysis on a FACS II or FACS IV (Becton Dickinson, Mountain View, Calif) dual laser fluorescent-activated cell sorter (see later). Stained cells were sterily separated from residual nonstaining marrow populations by virtue of fluorescent intensity; >98% of the positively sorted cells were T lymphocytes (assessed both by scatter distribution and by restaining with fluoresceinated Leu 4 pan-T MCAB). Cells with the fluorescent distribution of T lymphocytes were not observed when the separated nonstaining marrow population was either assessed for residual fluorescence or restained with Leu 4 MCAB. Sorted marrow populations were assessed for blastogenic responses and for growth of hematopoietic progenitors. Control cell populations were subjected to a similar FACS analysis but without selective cell separation; these cell suspensions were then similarly assessed for T lymphocytes and for growth of hematopoietic progenitors. Nonviable cells staining with propidium iodide were electronically excluded during all FACS analyses.

Assessment of Efficacy of T Lymphocyte Depletion

Multiparameter FACS analysis. Control and T cell-depleted marrow or peripheral blood populations were separately stained with varying combinations of three fluorescein-conjugated pan-T lymphocyte MCABs (Leu 1, Leu 4, or Leu 5).^{33,39,40} Stained cell populations were analyzed on a FACS II or FACS IV equipped with dual lasers, two independent fluorescence detection systems, a small-angle light scatter detector, and a propidium iodide detector.⁴¹ "List mode" data recording the scatter parameters and each of two fluorescence measurements for each cell were collected on 3×10^4 viable cells from each analyzed cell suspension and processed using a VAX 11/780 computer (Digital Equipment Corp, Maynard, Mass) to yield contour graphic plots depicting frequency distributions of cells with respect to scatter and fluorescence. Marrow T lymphocytes were thus delineated on the basis of relative autofluorescence, specific staining with fluorescein-conjugated pan-T cell MCABs, and both low-angle forward-angle light scatter and 90° light scatter.

Functional studies. Nonadherent marrow or peripheral blood MNCs (0.125 to 0.500×10^6 /mL) were suspended in round-bottom wells containing 0.2 mL RPMI 1640 medium supplemented with 25 mmol/L HEPES buffer, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% decomplemented pooled human serum.⁴² Stimulation with 10 μ L of either phytohemagglutinin ([PHA] 0.5 to 10.0 μ g/mL) or concanavalin A ([Con A] 10 to 100 μ g/mL) was carried out at 37 °C in 5% CO_2 for four to seven days of culture. Assays were performed in triplicate, and 1 μ Ci per well of ^3H -thymidine was added 18 hours before harvesting with a MASH II apparatus. MLCs (mixed leukocyte cultures) were car-

ried out for six days between 0.25 to 10.00×10^4 responder marrow nonadherent MNCs and similar numbers of irradiated (3,000 rad) stimulator cells from two to four separate allogeneic donors. Proliferation was expressed as the stimulation index (ratio of counts per minute of stimulated or experimental cultures to control cultures, E/C) or as the difference in counts per minute per 10^6 MCNs between stimulated and unstimulated (control) cultures (Δ cpm).

Microcytotoxicity and Indirect Immunofluorescence

Suspensions of buoyant human peripheral blood or marrow MNCs were assessed by microcytotoxicity and/or immunofluorescence according to previously described methods.^{31,36}

Suspension Cultures

These experiments were used to assess possible T lymphocyte replenishment in cultures of marrow MNCs previously depleted of T cells. Cultures containing 1 to 2×10^5 control or T cell-depleted (by either panning or FACS-gated separation) marrow MNCs were established in dishes containing IMDM, 30% FCS, $50 \mu\text{mol/L}$ mercaptoethanol, and 1 unit human urinary erythropoietin with or without 1% Mo CM. Quadruplicate plates were assessed for T lymphocytes by FACS analysis and fluorescent microscopy (using Leu 1, Leu 4, and Leu 5 pan-T cell MCABs) on day 0 and after three, seven, and 14 days incubation at 37°C in 5% CO_2 . Trypan blue dye exclusion assays and total cell counts were performed on representative cell aliquots from each set of cultures at the time of assessment for residual T lymphocytes.

Statistics

The two-sample unpaired Student's *t* test was used in statistical analysis of the data.⁴³ An errors-in-variables (structural) analysis⁴⁴ was used to statistically assess the linear relation between the percentage of marrow T lymphocytes and mitogenic stimulation indices after marrow T cell depletion.

RESULTS

In control experiments, peripheral blood and marrow MNCs were assessed for their susceptibility to complement-mediated antibody-dependent cytotoxicity in a fluorochromatic assay incorporating fluorescein diacetate (FDA) label and exclusion of ethidium bromide (EB). Leu 1 and TM1 pan-T lymphocyte MCABs were cytotoxic for 73% to 81% of peripheral blood MNCs and 10% to 12% of marrow MNCs. No cell cytotoxicity was noted after incubation with either diluted rabbit complement or murine ascites fluid, or after treatment with MCAB alone. In the presence of complement, >95% of peripheral blood erythrocyte-rosetting cells were killed after incubation with either Leu 1 or TM1, as assessed by the FDA-EB microcytotoxicity assay or by indirect immunofluorescence. The extent of peripheral blood lympholysis was unaffected by alteration in cell concentration (0.8 to 80.0×10^6 cells per milliliter) or the temperature (23°C v 37°C) or duration (2.5 hours v 1 hour) of the cytotoxicity assay, nor by variation in the marrow cell suspension medium (IMDM, human AB serum, or serum-free medium containing 5% human serum albumin).

Figure 1 depicts growth of erythroid (BFU-E),

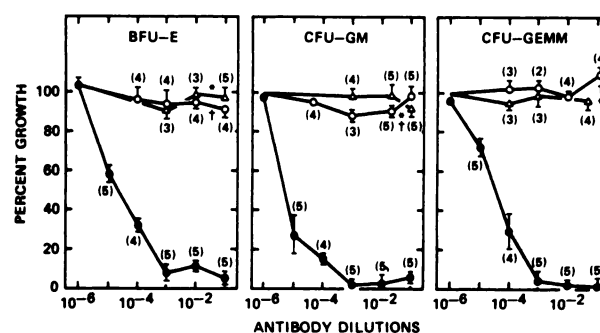


Fig 1. Effects of incubation with either of two pan-T cell MCABs (Leu 1 or TM1) or with an anti-HLA-DR MCAB (2.06) on in vitro growth of human marrow erythroid (BFU-E), granulocyte-macrophage (CFU-GM), and pluripotential (CFU-GEMM) hematopoietic progenitors. Marrow MNC suspensions were incubated with varying dilutions of MCAB with or without complement (C') before plating in methylcellulose cultures. Results are expressed as the percentage of control colony growth ($\pm$ SEM) after marrow cell incubation with tissue culture medium (IMDM) with or without C'. Numbers in parentheses reflect number of experiments. Control colony growth after marrow cell incubation with IMDM plus C' (mean $\pm$ SEM per 2×10^5 cells for four to five separate experiments) was 124 ± 18 BFU-E, 47 ± 9 CFU-GM, and 4.0 ± 1.0 CFU-GEMM. $\bullet$, Anti-HLA-DR (2.06) + C'; $\circ$, anti-T (Leu 1) + C'; Δ , anti-T (TM1) + C'; $\circ$, Leu 1 alone; $\dagger$, TM1 alone.

granulocyte-macrophage (CFU-GM), and multipotential (CFU-GEMM) hematopoietic progenitors after marrow cell incubation with varying concentrations of either Leu 1 or TM1 MCABs. Concomitant incubation of separate marrow cell suspensions with anti-HLA-DR (Ia-like) MCAB (2.06) plus complement was performed in each of this set of experiments. Minimal colony cytotoxicity was demonstrable when either pan-T lymphocyte antibody was used in the presence of complement. Leu 1 at a 1:10 dilution, for example, was associated with growth of $91\% \pm 6\%$ BFU-E, $98\% \pm 12\%$ CFU-GM, and $110\% \pm 16\%$ CFU-GEMM. Comparable colony growth was seen after incubation of marrow with TM1 plus complement at all antibody concentrations tested. No important effect on colony formation was noted after incubation with either antibody alone at concentrations as high as 1:10 (5 to $20 \mu\text{g}$ per 10^6 cells). In contrast, incubation of marrow with anti-HLA-DR (Ia-like) MCAB plus complement produced potent concentration-dependent, complement-mediated inhibition of all three hematopoietic progenitors (Fig 1). Sequential or simultaneous incubations of marrow cell suspensions with either Leu 1 or TM1 plus complement did not affect growth of hematopoietic progenitors. In further experiments, marrow was incubated with neuroaminidase-treated sheep red blood cells, and the nonrosetting marrow cell fraction subsequently treated with Leu 1 plus complement. BFU-E growth was $83\% \pm 8\%$, CFU-GM growth was $94\% \pm 10\%$, and CFU-GEMM growth was $90\% \pm 11\%$ of control.

Figure 2 shows the percentage of colony growth (corrected for concentration effect) after marrow MNC treatment with each of the four methods used for marrow T lymphocyte depletion. Colony growth after marrow incubation with anti-HLA-DR (Ia-like) MCAb 2.06 plus complement is also indicated. BFU-E growth ranged from 95% to 120% of control, CFU-GM growth from 90% to 108% of control, and CFU-GEMM growth from 89% to 111% of control.

Efficacy of Marrow T Cell Depletion

Multiparameter FACS analysis was next used to assess efficacy of the various methods aimed at marrow T lymphocyte depletion. Two-dimensional logarithmic contour graphic plots were used to depict frequency distributions of marrow cell subsets with respect to forward-angle light scatter and fluorescence (Figs 3 through 5). Dual argon ion lasers (488 and 582 nm) permitted further enhanced visualization of marrow T lymphocytes by virtue of delineating relative autofluorescence of various marrow subpopulations: T lymphocytes were found to have low autofluorescence relative to other marrow subsets, as reflected by an inability of the argon dye laser to excite red fluorescence in the T lymphocyte population at 582 nm (Fig 3A, ordinate); T cells stained with fluorescein-conjugated antisera still exhibited bright fluorescence, however, when excited at 488 nm (Fig 3A, abscissa).

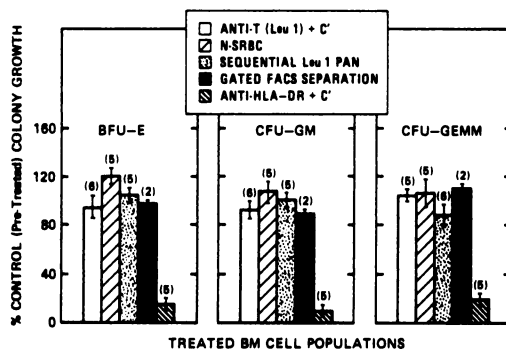


Fig 2. Effects of four separate methods of T lymphocyte depletion on in vitro growth of human marrow erythroid (BFU-E), granulocyte-macrophage (CFU-GM), and pluripotential (CFU-GEMM) hematopoietic progenitors. Marrow T cell depletion was performed by incubation of bone marrow (BM) MNCs with pan-T cell MCAb (Leu 1) plus complement (C'), by neuraminidase-treated sheep red blood cell (N-SRBC) gradient fractionation, by sequential immunoabsorbent panning procedures using Leu 1 antibody, and by FACS selective cell sorting and gated separation. Marrow colony growth was also assessed after marrow cell incubation with anti-HLA-DR monoclonal antibody (2.06) plus C'. Results are expressed as the percentage of control pretreated colony growth ($\pm$ SD) and are corrected for concentration effect after T cell depletion. Numbers in parentheses reflect number of experiments. Control pretreated colony growth (mean $\pm$ SEM per 2×10^5 marrow cells for three to five separate experiments) was 110 ± 17 BFU-E, 64 ± 10 CFU-GM, and 4.5 ± 1.5 CFU-GEMM.

The upper two panels of Fig 3 show a control marrow suspension (incubated with Leu 1 alone) and stained with a separate (Leu 5) pan-T lymphocyte MCAb. The marrow T cell population (arrows), here comprising 9% of all marrow MNCs, is easily depicted in the graphic display. The bottom two panels of Fig 3 display the same marrow cell suspension in the absence of fluoresceinated antibody; T cells are no longer visualized in the left-hand panel but can still be seen in the right-hand scatter plot (arrow).

The upper two panels of Fig 4 show marrow treated with sequential incubations of Leu 1 plus complement, and subsequently stained with fluoresceinated Leu 1 just before FACS analysis. T cells are no longer seen in the left-hand panel; cells with the scatter-fluorescent distribution of T cells, however, are still visualized on the scatter graph (arrow). These results suggest that cells with the scatter distribution of T lymphocytes remain in marrow after sequential treatment with Leu 1 plus complement; they further suggest that binding sites are no longer available to fluoresceinated Leu 1 after prior sequential treatment of marrow with Leu 1 antibody (similar scatter-fluorescent profiles were obtained with fluoresceinated Leu 1 after treatment of marrow with Leu 1 without complement). The presence of residual T cells in marrow after sequential Leu 1 treatment is further suggested by staining with a second pan-T MCAb (Leu 5) that is directed against different surface antigenic determinants (bottom two panels, Fig 4): cells with the plot distribution of T lymphocytes were easily discernable in both lower panels (arrows). In contrast, when nonrosetting marrow cells were stained with fluoresceinated Leu 1 or Leu 5, either before or after (Fig 5) treatment with Leu 1 plus complement, no cells with the fluorescent distribution or scatter characteristics of T lymphocytes could be visualized after FACS analysis.

Table 1 indicates efficacy of marrow T cell depletion (assessed by FACS analysis after staining with three separate pan-T MCABs) and extent of progenitor cell recovery after antibody- and complement-mediated cytotoxicity, solid-phase immunoabsorption (panning), fractionation of sheep cell rosetted marrow, or gated FACS separation. Cells staining positively for one or more of the fluorescent pan-T cell MCABs made up 8.4% to 9.5% of control nonadherent MNCs. T cells constituted 3.2% to 5.1% of marrow after single, sequential or combination treatment with Leu 1 or TM1 plus complement, and 1.5% to 2.2% of marrow after immune panning with Leu 1 with or without TM1. Fluorescent cells composed only 0.2% to 0.3% of marrow after sheep cell rosetting and only 0.05% of marrow after gated FACS separation. Marrow progenitor cell recovery averaged 71% to 94% of control

except after sheep cell rosette fractionation; colony recovery after the latter procedure averaged only 49% to 54% of control. T cells made up 59% to 73% of control peripheral blood MNCs and 0.8% to 2.8% of peripheral blood MNCs after sheep cell rosetting plus treatment with Leu 1 and complement ($n = 4$). A similar degree of peripheral blood T cell depletion was

observed in two experiments after FACS selective cell sorting and gated separation.

Efficacy of marrow T cell depletion after single, sequential, or combination treatment with Leu 1 or TM1 plus complement was unaffected by alterations in the temperature or duration of the cytotoxicity assay or by variation in cell concentration or composition of

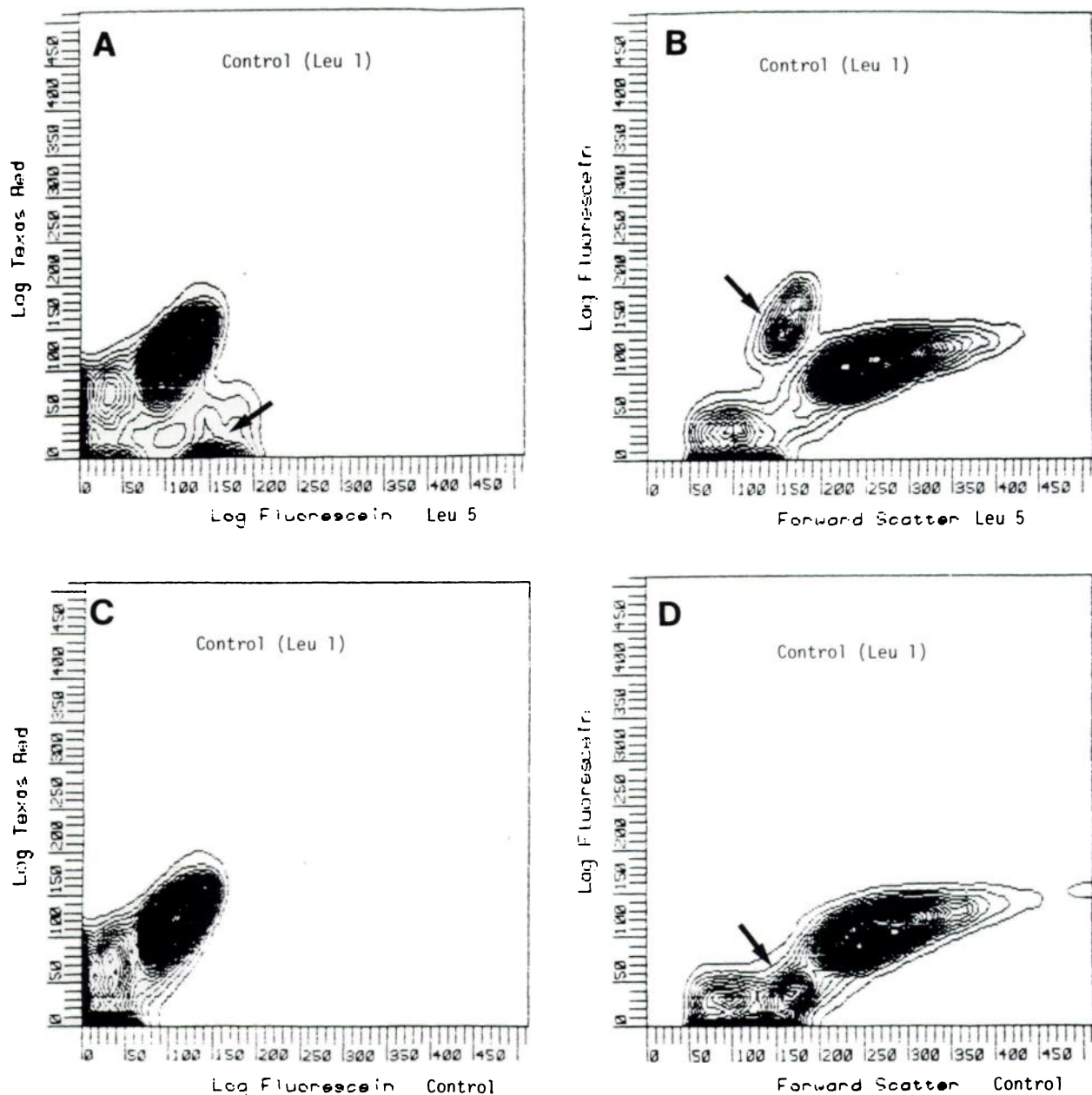


Fig 3. Logarithmic contour graphic plots of human marrow control MNC suspensions after FACS analysis. In panels A and C, cell autofluorescence (582 nm) is plotted on the ordinate and cell-antibody fluorescence (488 nm), after cell staining with fluorescent pan-T cell MCab (Leu 5) or with control medium, is plotted on the abscissa. In panels B and D, cell-antibody fluorescence is plotted on the ordinate and cell scatter (determined by cell volume and cell size) is plotted on the abscissa. Marrow cell suspensions in all four panels were preincubated with Leu 1 MCab without complement before FACS analysis. Arrows indicate marrow T cells in either the fluorescent (panel A) or scatter (panels B and D) plots. Aliquots from the same marrow cell suspension were analyzed in all four panels.

the marrow cell suspension medium. Three separate lots of Leu 1 and five separate lots of rabbit complement failed to provide increased T lymphocyte depletion. Threefold to sixfold increases in concentration of rabbit complement (after incubation with either Leu 1 or TM1) resulted in a further 20% to 34% decrease in

progenitor cell recovery with no increase in T cell depletion. Comparable results were obtained in two experiments, after sequential treatment with Leu 1 plus complement, when residual T lymphocytes were assessed using either fluorescent Leu 4 MCAB ($3.8\% \pm 1.1\%$) or fluorescent goat anti-mouse im-

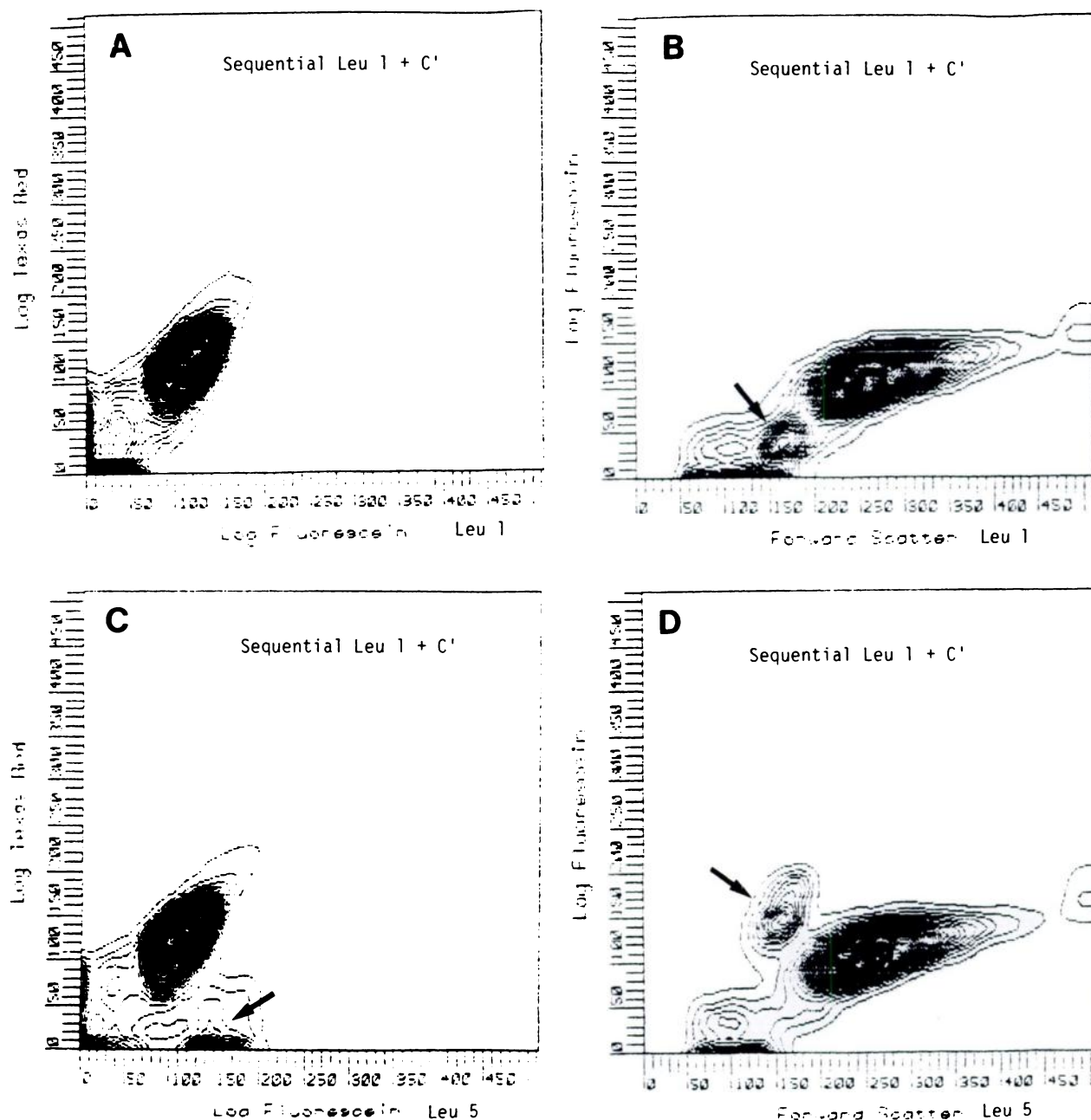


Fig 4. Logarithmic contour graphic plots of human marrow MNC suspensions after FACS analysis. Marrow cell suspensions in all four panels were preincubated with Leu 1 MCAB plus complement (C') before FACS analysis. In panels A and C, cell autofluorescence is plotted on the ordinate and cell-antibody fluorescence (after cell staining with either fluorescent pan-T cell Leu 1 antibody or fluorescent pan-T cell Leu 5 antibody) is plotted on the abscissa. In panels B and D, specific cell-antibody (either Leu 1 or Leu 5) fluorescence is plotted on the ordinate and cell scatter on the abscissa. Arrows indicate marrow T cells in either the fluorescent (panel C) or scatter (panels B and D) plots. Aliquots from the same marrow cell suspension were analyzed in all four panels.

noglobulin ($3.1\% \pm 0.9\%$). The presence or absence of 0.1% sodium azide in the Leu 1 cytotoxicity assay had no effect on T cell depletion. The use of goat anti-mouse immunoglobulin in an antiglobulin-enhanced cytotoxicity assay with Leu 1 plus complement also did not increase the efficacy of marrow T cell depletion.

Marrow Lymphocyte Blastogenic Responses After T Cell Depletion

Marrow lymphocyte blastogenic responses (after stimulation with PHA, Con A, or allogeneic-irradiated MNCs) were assessed in control and treated marrow

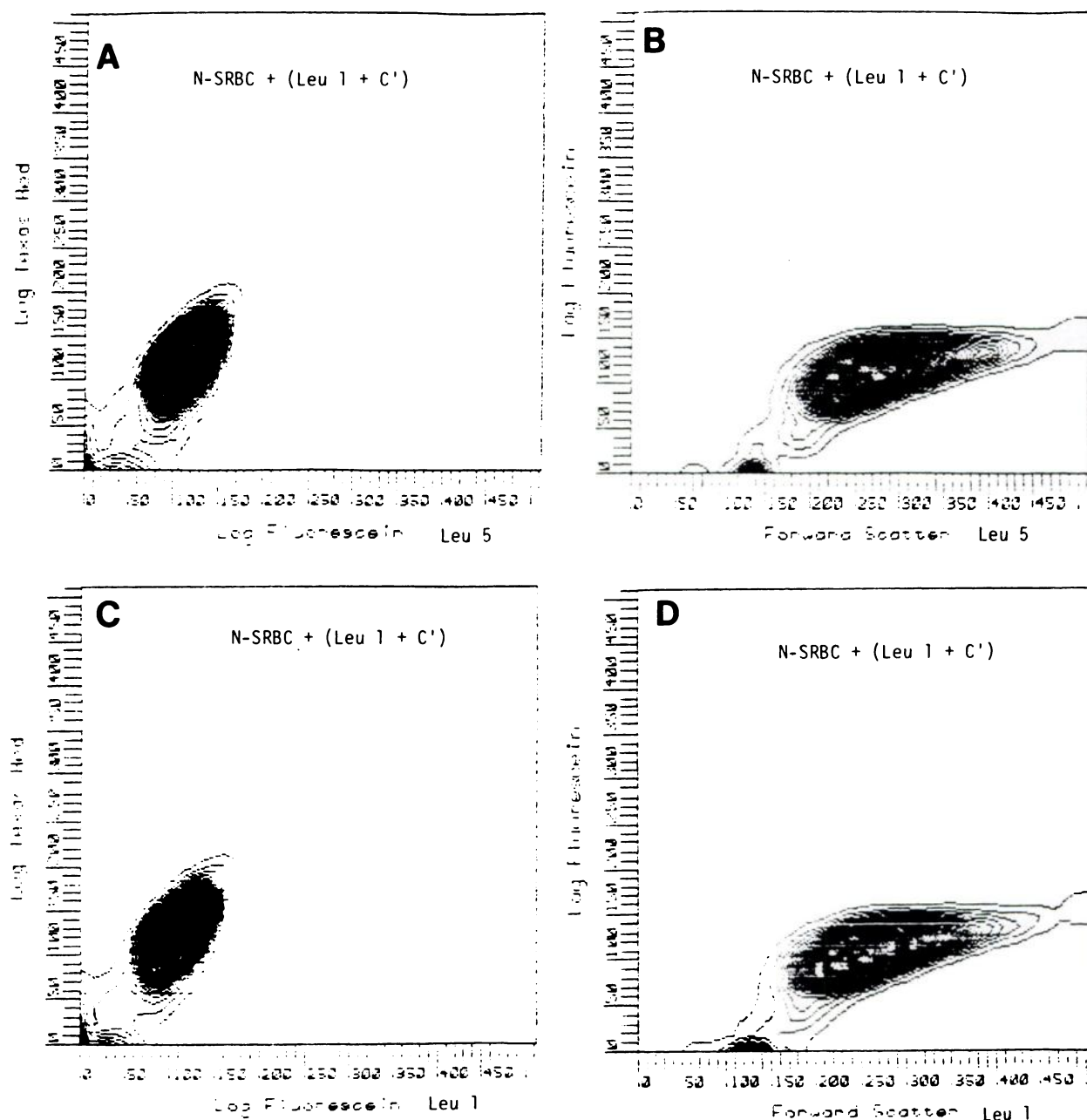


Fig 5. Logarithmic contour graphic plots of human marrow MNC suspensions after FACS analysis. Marrow cell suspensions in all four panels were first incubated with neuraminidase-treated sheep red blood cells (N-SRBC). The nonrosetting marrow cell fraction was subsequently incubated with pan-T cell MCab (Leu 1) plus complement (C') before FACS analysis. In panels A and C, cell autofluorescence is again plotted on the ordinate and specific cell-antibody fluorescence is plotted on the abscissa. In panels B and D, specific cell-antibody (either Leu 5 or Leu 1) fluorescence is plotted on the ordinate and cell scatter on the abscissa. Marrow T cells cannot be visualized in either the fluorescent (panels A and C) or the scatter (panels B and D) plots. Absence of visualized T cells in the scatter plots suggests that N-SRBC interference with MCab-receptor binding is unlikely.

Table 1. Residual Marrow T Lymphocytes and Hematopoietic Progenitor Cell Recovery After Four Separate Methods of Human Marrow T Cell Depletion

Cell population	Marrow T Lymphocytes (%)			Progenitor Cell Recovery (%)	
	Leu 1	Leu 4	Leu 5	CFU-GM	BFU-E
Control marrow (n = 6)	8.4 ± 1.1	9.5 ± 1.7	9.1 ± 1.3		
Pan-T-MCAB + C'					
Leu 1 (n = 8)	ND	4.8 ± 0.8	4.3 ± 1.1	94 ± 10	90 ± 8
TM1 (n = 4)	5.1 ± 1.4	4.4 ± 1.4	ND		
Leu 1→Leu 1 (n = 5)	ND	3.9 ± 0.7	3.2 ± 1.0	88 ± 6	79 ± 9
Leu 1 + TM1 (n = 3)	ND	3.2 ± 0.6	2.8 ± 0.8	87 ± 7	93 ± 8
	$P < .01$		$P < .01$		
Immune panning					
Leu 1 (n = 5)	1.8 ± 0.6	2.2 ± 0.5	1.6 ± 0.3	79 ± 14	85 ± 9
Leu 1→Leu 1 (n = 4)	1.6 ± 0.5	1.9 ± 0.8	ND	71 ± 10	79 ± 8
Leu 1→TM1 (n = 3)	1.5 ± 0.4	2.0 ± 0.7	1.9 ± 0.4		
	$P < .01$		$P < .01$		
N-SRBC gradient fractionation (n = 5)	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.2	49 ± 6	54 ± 7
	$P < .05$				
Gated FACS Separation (n = 3)	ND	0.05 ± 0.03	ND	84 ± 5	87 ± 6

All values are mean ± SD. CFU-GM, granulocyte-monocyte colony-forming units; BFU-E, burst-forming unit-erythroid; MCAB, monoclonal antibody; C', complement; N-SRBC, neuraminidase-treated sheep red blood cells; FACS, fluorescent-activated cell sorter; n, number of experiments; ND, not done. Only representative *P* values are indicated.

Marrow T lymphocytes were assessed by FACS analysis after independent staining with three separate fluoresceinated pan-T cell monoclonal antibodies (Leu 1, Leu 4, or Leu 5). Progenitor cell recovery was calculated from the total number of marrow MNCs after each depletion procedure, and from the colony-plating efficiency per 2×10^5 marrow cells. Marrow T cell depletion was attempted by single, sequential (→), or combination (+) treatment with two pan-T cell MCAB's (Leu 1 or TM1) plus C', by either single or sequential (→) immunoabsorbent panning procedures using either Leu 1 or TM1, by N-SRBC gradient fractionation, or by FACS selective cell sorting and gated separation.

nonadherent cell populations to gauge functional T lymphocyte activity after T cell depletion (Table 2). Results are expressed as stimulation indices using optimal mitogen concentrations; qualitatively similar responses were noted over a tenfold to 20-fold range of mitogen concentrations. Adherent marrow cell fractionation was necessary for maximal stimulation response. As indicated in Table 2, stimulation indices fell progressively, relative to control, as marrow T cell depletion was attempted with either sequential Leu 1 plus complement, sequential immune panning, or sheep cell rosette fractionation. These functional studies of treated marrow populations are thus in accord with the prior FACS analyses concerning efficacy of marrow T lymphocyte depletion, as demonstrated by the linear correlation between the percentage of marrow T cells (assessed by cell sorter analysis) and mitogenic stimulation indices after all four methods of T cell depletion (Fig 6, $P < .001$ by an errors-in-variables analysis, for each of the three separate mitogen curves). Peripheral blood MNC stimulation indices using optimal concentrations of either PHA or Con A fell from control values of 56 to 84 to 0.6 to 1.6 after T cell depletion by sheep cell rosette fractionation plus treatment with Leu 1 plus complement (n = 3).

Effects of Autologous T Cells and Monocytes on Growth of Marrow Hematopoietic Progenitors

To further assess T lymphocyte modulation of in vitro marrow hematopoietic progenitor cell growth and differentiation, control marrow, as well as marrow depleted of T cells by either sheep cell rosette fractionation or gated FACS separation, was assayed for growth of BFU-E and CFU-GM in the presence and absence of Mo CM, a conditioned medium derived from a T lymphocyte cell line (Table 3). CFU-GM growth was also assessed after T cell depletion using human placental-conditioned medium as a substitute source of colony-stimulating activity (CSA). Bone marrow T lymphocyte depletion did not alter BFU-E growth either in the presence or in the absence of Mo CM, nor did it alter growth of CFU-GM when human placental CM was used, rather than Mo CM, as an alternate source of CSA (Table 3). In further experiments, the effect of marrow T cell depletion on in vitro growth of erythroid progenitors was assessed under conditions of reduced FCS concentrations to optimize detection of BPA. In control experiments, the addition of 1% Mo CM (a previously described source of BPA⁴⁵) caused a threefold elevation in BFU-E growth

Table 2. Effects of T Lymphocyte Depletion on Marrow Blastogenic Response to Con A, PHA, and Allogeneic-Irradiated Cells

		Con A (10 µg/mL)		PHA (10 µg/mL)		MLC (2.5 × 10 ⁴ Cells)	
Control BM							
1 × 10 ⁵ cells		5.9 ± 1.3		5.3 ± 1.1		15.7 ± 2.3	
5 × 10 ⁴ cells		7.6 ± 0.9		6.6 ± 0.8		14.8 ± 1.9	
Sequential Leu 1 + C'	<i>P</i> < .02	3.9 ± 0.9	<i>P</i> < .01	4.0 ± 0.9	<i>P</i> < .02	7.6 ± 1.1	
1 × 10 ⁵ cells		5.7 ± 0.8		3.7 ± 0.6		9.0 ± 1.6	
5 × 10 ⁴ cells							
Sequential Leu 1 Panning	<i>P</i> < .01	1.6 ± 0.7	<i>P</i> < .01	2.1 ± 0.7	<i>P</i> < .01	2.9 ± 1.1	
1 × 10 ⁵ cells		2.4 ± 0.6		1.7 ± 0.4		3.5 ± 1.0	
5 × 10 ⁴ cells							
N-SRBC Rosetting	<i>P</i> < .01	1.1 ± 0.6	<i>P</i> < .02	1.2 ± 0.5	<i>P</i> < .01	1.3 ± 0.8	
1 × 10 ⁵ cells		0.8 ± 0.4		0.7 ± 0.6		1.2 ± 0.4	
5 × 10 ⁴ cells							

All values are mean ± SD from three to five separate experiments. Responses are expressed as stimulation indices (E/C, ratio of cpm of stimulated or experimental cultures to control cultures) using optimal mitogen concentrations. Varying concentrations (0.125 × 10⁶–0.5 × 10⁶/mL) of control and T cell-depleted nonadherent buoyant MNCs were established in triplicate cultures before the addition of 10 µL of mitogen or control fluids. Optimal marrow cell stimulation was found at four days of culture for both PHA and Con A and at six days of culture for the MLC response. Mitogen dose responses were determined for 0.5 to 10 µg/mL of PHA and for 10 to 100 µg/mL of Con A. Each marrow suspension was assessed in MLC against irradiated (3,000 rad) stimulator cells from two to four separate allogeneic donors. Only representative *P* values are indicated.

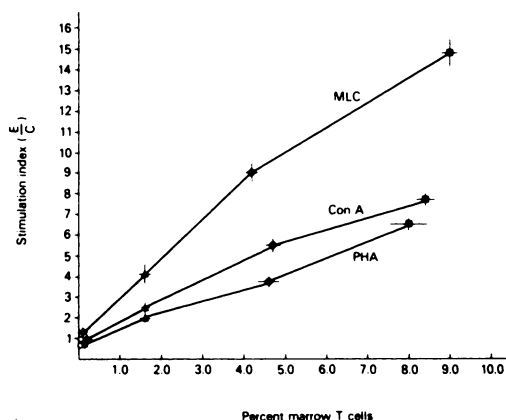


Fig 6. Percentage of marrow T lymphocytes and marrow blastogenic responses to PHA, Con A, and allogeneic-irradiated peripheral blood MNCs after four separate methods of marrow T cell depletion. Marrow T lymphocytes were assessed by FACS analysis after staining with two to three separate fluoresceinated pan-T cell MCAs as described in Materials and Methods and in Table 1. Marrow blastogenic responses were determined as noted in Materials and Methods and in Table 2. Marrow T cell depletion was attempted concurrently by sequential treatment with Leu 1 MCA plus complement (C'), by sequential immunoabsorbent panning with Leu 1, by neuraminidase-treated sheep red blood cell (N-SRBC) gradient fractionation, or by FACS selective cell sorting, before functional assessment. Results are expressed as (mean ± SEM) for four to six separate experiments (*P* < .001 in assessing the linear coefficient between stimulation indices ($\frac{E}{C}$) and percentage of marrow T cells for each of the three curves). ■, Control; ♦, sequential Leu 1 + C'; ▲, sequential pan with Leu 1; ●, N-SRBC; ○, gated FACS.

when added to marrow cultures containing erythropoietin plus 15% FCS; T cell depletion under these conditions again failed to alter growth of human BFU-E (Table 3). Marrow T cell depletion did not affect BFU-E or CFU-GM size and cytologic composition (assessed by micropipet selection and histochemical staining) nor did it affect latency of colony development (time from plating to documentation of maximal size and number of either BFU-E or CFU-GM). Numbers of large (>16 clusters) "immature" marrow BFU-E were also not affected by T cell depletion. Marrow monocyte depletion (NAB) or marrow depletion of both T cells and monocytes (T_F) in four separate experiments had no significant effect on in vitro growth of BFU-E (Fig 7). In three further experiments, BFU-E growth was assessed before and after the addition of 1% to 15% autologous marrow T cells to cultures containing nonadherent marrow depleted of T cells by panning; marrow T lymphocytes had a negligible effect on BFU-E growth (*P* > .1 for T_F + 1%, T_F + 5%, T_F + 10%, or T_F + 15% relative to either T_F or NAB marrow alone or control (*) marrow target cells, Fig 7). Similar results were obtained when as many as 50 to 100 × 10³ T cells or monocytes were added back to 10⁵ nonadherent T cell-depleted marrow target cells in the presence or absence of Mo CM (*n* = 3).

Suspension cultures of control and T cell-depleted marrow MNCs were assessed over 14 days of incubation for evidence of possible in vitro T lymphocyte repletion (Table 4). Marrow cells were incubated both

Table 3. Effects of Human Marrow T Lymphocyte Depletion on In Vitro Growth of Erythroid and Granulocyte-Macrophage Progenitors in the Presence and Absence of Mo-Conditioned Medium

	Mo	Ep	HPCM	Colonies/ 2×10^5 Marrow Cells		
				BFU-E		CFU-GM
				30% FCS	15% FCS	30% FCS
Control BM	+	+	—	132 $\pm$ 18	82 $\pm$ 11	53 $\pm$ 6
N-SRBC BM	+	+	—	153 $\pm$ 23	95 $\pm$ 6	58 $\pm$ 9
FACS sorted BM	+	+	—	143 $\pm$ 17	87 $\pm$ 8	49 $\pm$ 8
Control BM	—	+	—	84 $\pm$ 14	27 $\pm$ 7	1 $\pm$ 3
N-SRBC BM	—	+	—	92 $\pm$ 11	38 $\pm$ 5	2 $\pm$ 2
FACS Sorted BM	—	+	—	80 $\pm$ 6	30 $\pm$ 7	2 $\pm$ 1
Control BM	—	—	+	0	0	124 $\pm$ 19
N-SRBC BM	—	—	+	0	0	105 $\pm$ 11
FACS Sorted BM	—	—	+	0	0	111 $\pm$ 14

Colony growth was assessed from control marrow MNCs and from marrow depleted of T lymphocytes by either N-SRBC gradient fractionation or by FACS selective cell sorting and gated separation.

All values are mean $\pm$ SD from four separate experiments. BFU-E, burst-forming unit-erythroid; CFU-GM, granulocyte-monocyte colony forming units; N-SRBC, neuraminidase-treated sheep red blood cells; FACS, fluorescent-activated cell sorter; Mo, a conditioned medium derived from a T lymphoblast cell line; Ep, erythropoietin; HPCM, human placental-conditioned medium—an alternative source of colony-stimulating activity for the in vitro growth of CFU-GM; BM, bone marrow; FCS, fetal calf serum.

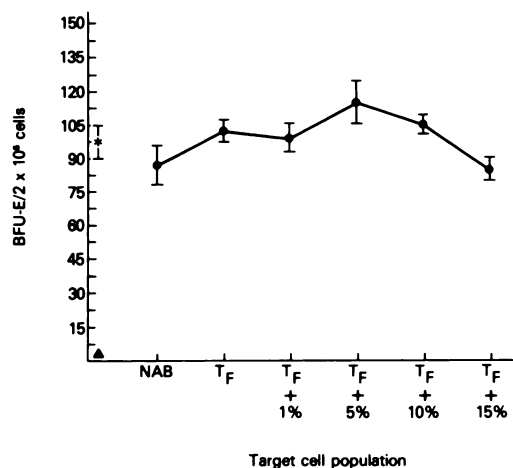


Fig 7. Marrow erythroid progenitor (BFU-E) growth in vitro before and after the addition of 1% to 15% marrow autologous T cells to nonadherent T cell-depleted human marrow cultures. Marrow MNCs were first depleted of monocytes by two consecutive 90-minute adherence procedures. Nonadherent buoyant (NAB) marrow MNCs were either assayed directly for BFU-E or first depleted of T lymphocytes by sequential immunoabsorbent panning with Leu 1 pan-T cell MCAb. Enriched marrow T cells (>95% positive staining by Leu 1 and/or Leu 5 pan-T cell MCAs) were recovered from the bound MNC fraction of panned marrow and added back to 2×10^5 nonadherent T-free (T_F) marrow mononuclear cells before assay for BFU-E. Results are expressed as mean ($\pm$ SD) BFU-E colonies per 2×10^5 marrow MNCs for three to four separate experiments. Enriched marrow T lymphocytes (Δ) yielded virtually no erythroid colonies. Asterisk (*) denotes number of erythroid colonies per 2×10^5 marrow MNCs before monocyte and T cell depletion.

in the presence and in the absence of Mo CM. Plates were assessed for T lymphocytes by indirect immunofluorescence microscopy using two separate pan-T cell MCAs (Leu 1 and Leu 5). No statistically significant increase in T lymphocytes was noted after 14 days of culture in marrow previously depleted of T cells and grown in the absence of Mo CM. Small increases in total numbers of T cells were seen after 14 days of incubation in T cell-depleted marrow cultures grown in the presence of Mo CM ($0.01 < P < .05$); T lymphocytes in these latter cultures, however, still composed only 1.5% of the total marrow MNC population. Similar results were obtained when T lymphocytes were enumerated by fluoresceinated Leu 1 and Leu 5 antibodies by FACS analysis.

Effects of Autologous T Cells and Monocytes on Growth of Peripheral Blood Erythroid Progenitors

The effect of T lymphocyte depletion on peripheral blood BFU-E was assessed after incubating blood MNC suspensions with N-SRBC and treating the nonrosetting cell fraction with Leu 1 MCAb plus complement (Table 5A). In four separate experiments, peripheral blood BFU-E per 10^5 T-depleted target cells were 89%, 87%, 78%, and 169% (mean, 106%) of expected. T depletion similarly failed to affect growth of peripheral blood BFU-E when plated cells were assessed at FCS concentrations as low as 10%. In contrast to human marrow, monocyte depletion from peripheral blood resulted in >90% inhibition of BFU-E (Table 5B). In further experiments, varying concentrations of purified autologous blood T cells or monocytes were added back to nonadherent T cell-depleted

peripheral blood MNCs ("null cells"). In five control experiments, BFU-E growth from nonadherent T cell-depleted peripheral blood MNCs ranged from 5% to 16% ($8\% \pm 4\%$, mean $\pm$ SD) of expected (Table 6). The addition of as many as 150×10^3 T cells to 10^5 autologous nonadherent T cell-depleted blood "null" cells stimulated only a modest increment in BFU-E growth (18% to 30% of expected values as determined by simultaneous culture of autologous unfractionated blood mononuclear cells) (Table 6). BFU-E growth was restored to 82% to 94% of expected values ($n = 5$) after T-depleted nonadherent blood target cells were cultured with 50 to 100×10^3 autologous peripheral blood monocytes.

DISCUSSION

Serial fractionation with sheep red blood cells, and complement-mediated cytolysis using either heteroantisera or MCAs have been proposed as promising alternative approaches for donor marrow T lymphocyte eradication in an effort to ameliorate GVHD and to facilitate marrow transplantation across either major or minor histocompatibility barriers.^{3,5,6,46} In addition to these standardized methods for T cell depletion, we have adapted the principles of solid-phase immunoabsorption and multiparameter FACS separation to more clearly delineate the efficacy and possible extent of human marrow T cell depletion, and

to further assess the role of accessory cells in regulation of human hematopoiesis. Multiparameter FACS analysis using a dual laser cell sorter permitted careful delineation of marrow T lymphocyte populations.⁴¹ Measurement of cell autofluorescence and forward-angle light scatter, as well as detection of specific cell fluorescence after staining with three different pan-T cell MCAs,^{33,39,40} permitted careful comparative assessment of the small numbers of residual T cells remaining after various attempts at marrow T lymphocyte depletion. Accurate delineation of small populations of residual marrow T lymphocytes is an important consideration in assessing the likelihood of GVHD after attempted donor marrow T cell depletion: Korngold and Sprent⁶ have shown, for example, that as little as 0.3% T cell contamination of donor marrow (after T cell depletion with anti-Thy 1.2 serum plus complement) still produces lethal GVHD in the majority of murine recipients after infusion of allogeneic MHC identical marrow.⁶ Recent clinical studies also indicate that very small numbers of residual donor marrow T lymphocytes are still capable of mediating GVHD after histoincompatible human marrow transplantation.^{16,47}

Comparable results were obtained in our studies when marrow T lymphocytes were assessed on the FACS with any one of three separate pan-T cell MCAs (Table 1). These results correlated closely

Table 4. Assessment of T Lymphocyte Repletion in Marrow Cultures Previously Depleted of T Cells by Solid-Phase Immunoabsorption

	BM Cells ($\pm$ Mo CM)		T Lymphocytes		Viability (Trypan Blue) Percent
			Percent	Total Number ($\times 10^4$)	
Day 0 ($n = 3$)	CON	—	9.8 ± 1.2	1.96 ± 0.30	96
	CON	+	9.4 ± 1.3	1.85 ± 0.40	94
	T _F	—	1.4 ± 0.4	0.28 ± 0.06	96
	T _F	+	1.2 ± 0.5	0.24 ± 0.11	93
Day 3 ($n = 2$)	CON	—	8.5 ± 1.1	1.64 ± 0.41 ($P > .05$)	89
	CON	+	7.3 ± 0.8	1.76 ± 0.33 ($P > .1$)	93
	T _F	—	1.1 ± 0.3	0.16 ± 0.06 ($P > .05$)	91
	F	+	0.9 ± 0.4	0.18 ± 0.05 ($P > .1$)	94
Day 7 ($n = 2$)	CON	—	8.1 ± 1.5	1.78 ± 0.52 ($P > .1$)	96
	CON	+	6.8 ± 1.2	1.94 ± 0.44 ($P > .1$)	92
	T _F	—	0.8 ± 0.4	0.21 ± 0.05 ($P > .1$)	94
	T _F	+	0.9 ± 0.4	0.26 ± 0.11 ($P > .1$)	91
Day 14 ($n = 3$)	CON	—	8.8 ± 1.6	2.25 ± 0.47 ($P > .05$)	94
	CON	+	7.6 ± 1.4	2.80 ± 0.58 ($0.01 < P < .05$)	92
	T _F	—	1.2 ± 0.5	0.31 ± 0.06 ($P > .1$)	92
	T _F	+	1.5 ± 0.4	0.39 ± 0.08 ($0.01 < P < .05$)	93

Control and T cell-depleted marrow suspensions were assessed over 14 days of culture for evidence of possible in vitro T lymphocyte repletion. Cultures were established both in the presence and in the absence of Mo CM. T lymphocytes were enumerated by indirect immunofluorescence microscopy using two separate fluoresceinated pan-T cell monoclonal antibodies (Leu 1 and 5). All P values are expressed relative to the respective cell suspension on day 0.

All values are mean $\pm$ SD. Mo CM, a conditioned medium derived from a T lymphoblast cell line; CON, control marrow buoyant mononuclear cells; T_F, marrow mononuclear cells depleted of T lymphocytes by a sequential immunoabsorbent panning procedure using Leu 1 monoclonal antibody; n , number of experiments; BM, bone marrow.

Table 5. Effects of T Lymphocyte or Monocyte Depletion on In Vitro Growth of Human Peripheral Blood BFU-E

Control MNCs (%)	Depleted MNCs (%)	Control MNC	BFU-E/10 ⁵ MNCs	
			Depleted MNCs	
			Observed	Expected
(A) T lymphocytes				
64	0.8	35 ± 3	84 ± 8	94
59	2.8	17 ± 1	34 ± 5	39
73	1.2	9 ± 2	25 ± 3	32
65	2.5	13 ± 3	59 ± 4	35
(B) Monocytes				
22	1.5	24 ± 2	3 ± 1	30
25	1.5	18 ± 3	2 ± 1	24
16	1.0	37 ± 5	4 ± 2	44
23	2.0	17 ± 2	0	22

All values are means ± SD from four separate experiments, each plated in quadruplicate. MNC, mononuclear cells; BFU-E, burst-forming unit-erythroid. T lymphocyte depletion was performed by N-SRBC density gradient centrifugation and treating the nonrosetting cell fraction with Leu 1 plus complement. Monocyte depletion was performed by two consecutive 90-minute adherence procedures. Residual T cells and monocytes were enumerated by immunofluorescence microscopy with Leu 4 and Leu M3 monoclonal antibodies, respectively. The expected number of BFU-Es per 10⁵ T cell-depleted target cells was calculated by: (BFU-E/10⁵ control MNC) × 100 ÷ [100 - (% T cells in control MNC - % T cells in T-depleted MNCs)]. In three further experiments, no significant differences in BFU-E growth were observed between control and T-depleted MNCs when the T-depleted targets were resuspended, before plating, to original volumes based on pretreated cell counts. The expected number of BFU-Es per 10⁵ monocyte-depleted target cells was calculated by: (BFU-E/10⁵ control MNC) × 100 ÷ [100 - (% monocytes in control MNC - % monocytes in monocyte-depleted MNCs)].

with marrow T cell functional activity as assessed by proliferative responses to three different mitogens and alloantigens (Fig 6). T lymphocytes constituted 8.4% to 9.5% of our control nonadherent marrow MNC population; this measurement is in close accord with prior estimates of T lymphocytes in human marrow MNC suspensions.^{48,49} Antibody-dependent T cell cytotoxicity was not appreciably enhanced by sequen-

tial or combination treatment with MCABs, nor by wide variation in complement lot or concentration, assay conditions, or monoclonal reagents. An antiglobulin-enhanced cytotoxicity technique (used to enhance complement fixation and cell cytotoxicity³⁷) also did not increase the efficacy of Leu 1-mediated marrow T cell depletion. Both TM1 and Leu 1 MCABs effectively lysed >95% of peripheral blood T lymphocytes under assay conditions virtually identical to those used for marrow T cell depletion. The explanation for this disparity in relative cytotoxic effect between peripheral blood and marrow T lymphocytes is not clear. In preliminary experiments, Leu 1 surface antigen distribution and density did not appear to differ between peripheral blood and marrow T cells (T. Kipps, unpublished observations, November 1982). Differential susceptibility to complement-mediated lysis may be a reflection of further differences between marrow and peripheral blood T lymphocytes. Fauci⁴⁸ and Gale et al⁵⁰ have described different proportions of functionally distinct lymphocyte subpopulations in the peripheral blood and marrow of normal individuals. Abdou et al⁴⁹ have noted that human marrow T cells are more efficient than peripheral blood T cells in inhibiting immunoglobulin synthesis from peripheral blood B cells, and Janossy et al⁵¹ have indicated that marrow T cells show mostly the suppressor/cytotoxic phenotype in contradistinction to peripheral blood T cells, which show a helper-suppressor/cytotoxic ratio of approximately 2:1.

Neither of the two pan-T cell MCABs used in our studies demonstrate significant mitogenic activity in standard proliferation assays.⁵² Diminished antibody mitogenic activity and an inability to induce T lymphocyte proliferation are properties that should facilitate effective antibody-dependent, complement-mediated T cell lysis and marrow T cell depletion.¹⁶ Marrow T cell depletion with Leu 1 or TM1 was not affected by the presence or absence of sodium azide in the suspen-

Table 6. Effects of T Lymphocytes or Monocytes on In Vitro Growth of Human Peripheral Blood BFU-E

BFU-E/10 ⁵ Control MNCs		Observed BFU-E After Addition of Either T Cells or MØ to 10 ⁵ MNC Depleted of Both T Cells and MØ							
		BFU-E/10 ⁵ T Cell and MØ-Depleted MNCs		50 × 10 ³		100 × 10 ³		150 × 10 ³	
				T Cells	MØ	T Cells	MØ	T Cells	MØ
24 ± 5	17 ± 3	114	23 ± 5 (20)	74 ± 8 (65)	27 ± 7 (23)	98 ± 7 (86)	33 ± 5 (28)	91 ± 11 (80)	
17 ± 6	9 ± 2	154	18 ± 4 (11)	115 ± 10 (75)	31 ± 5 (20)	143 ± 11 (94)	29 ± 7 (18)	119 ± 12 (77)	
9 ± 2	2 ± 1	39	8 ± 3 (20)	22 ± 6 (56)	11 ± 4 (28)	32 ± 5 (82)	12 ± 5 (30)	31 ± 6 (79)	
31 ± 8	11 ± 4	221	10 ± 5 (4)	152 ± 15 (69)	33 ± 8 (15)	184 ± 17 (83)	41 ± 10 (18)	187 ± 17 (85)	
26 ± 7	23 ± 7	236	21 ± 8 (9)	149 ± 21 (63)	30 ± 9 (13)	221 ± 21 (93)	63 ± 8 (26)	209 ± 19 (89)	

All values are means ± SD from five separate experiments, each plated in triplicate. Varying numbers of purified peripheral blood T cells or monocytes (MØ) were added to methylcellulose cultures containing 10⁵ peripheral blood target MNCs depleted of both monocytes and T cells. Monocyte and T cell depletion was performed as described in Table 5. Values in parentheses represent the percentage of expected colony growth.

The expected number of BFU-Es per 10⁵ T cells and monocyte-depleted target cells was calculated by: (BFU-E/10⁵ control MNC) × 100 ÷ [100 - (% monocytes and T cells in control MNC)].

sion medium nor by lowering the temperature of the first stage of the cytotoxicity procedure to 4 °C; comparable results were obtained when residual marrow T cells were assessed, after treatment with Leu 1 plus complement, with either fluorescent Leu 4 antibody or with a fluorescent goat anti-mouse immunoglobulin. These results suggest that MCAB-induced modulation of T cell surface antigens did not provide a major impediment to successful complement-mediated lysis.⁵³

Granger et al⁵⁴ have reported effective elimination of T lymphocytes from human bone marrow using a combination of OKT3 and OKT11A MCABs plus complement. T lymphocytes were reduced from 20% in control marrow suspensions to 6% in marrow treated with a combination of OKT3 and OKT11A plus complement. Possible surface membrane antigenic modulation was not assessed in this study, however, and nonviable cells were not excluded from analysis. Reinherz et al¹⁶ have described a single case wherein a reduction in donor marrow T lymphocytes from 10% to <0.1% was achieved after *in vitro* sequential treatment of marrow with anti-T12 MCAB plus complement. No functional assessments of T cell proliferative response were performed with the donor marrow after attempted T cell depletion. Interestingly, acute GVHD still developed in this haplotype-mismatched patient ten days after transplantation. In a preliminary report, Spruce et al⁴⁷ have described the occurrence of GVHD after haplotype-mismatched marrow transplantation in three patients despite prior incubation of donor marrow with SCI pan-T lymphocyte MCAB plus rabbit complement. Complement-mediated cytolysis using either heteroantisera or MCABs may still prove to be an effective method for donor marrow T cell eradication and suppression of GVHD after marrow transplantation. Our results, as well as the results of preliminary clinical trials,^{16,47} suggest, however, that caution must be exercised in applying the results of animal cytolytic T cell depletion experiments to human marrow transplantation. Solid-phase immunoabsorption ("panning") of human bone marrow is not easily applicable to the large volume of marrow that would need to be processed in clinical transplantation, and the extent of T cell depletion with this procedure may not be optimal. Density-gradient fractionation of sheep red blood cell rosetted marrow results in marked T lymphocyte depletion (0.2%) but poor hematopoietic progenitor cell recovery (49% to 54%). FACS-gated separation techniques also cause marked T cell depletion (0.05%) but with improved (84% to 87%) progenitor cell recovery. This latter method, however, entails considerable expense and logistic difficulties; a maximal cell sorting and separation rate of only 20×10^6

marrow MNCs per hour would provide for considerable delay and difficulties with cell viability during marrow processing. Recent progress in bone marrow separation and concentration techniques using an IBM cell processor,⁵⁵ a Sepharose cellular immunoabsorption column,⁵⁶ or ricin-conjugated MCABs,⁵⁷ may obviate some of the difficulties encountered with the above methods of marrow T cell depletion.

Treatment of allogeneic bone marrow cells with either antithymocyte globulin or pan-T cell MCABs before marrow transplantation has not generally interfered with successful bone marrow engraftment.^{5,6,46} These observations suggest that human pluripotential stem cells lack thymocyte or mature T lymphocyte surface antigens. Our results (Figs 1 and 2) provide more direct evidence that T lymphocyte surface antigenic determinants are not expressed on the majority of human multipotent (CFU-GEMM) stem cells. Murine multipotent hematopoietic progenitor cells (CFU-S) have been reported to lack Ia antigens,⁵⁸ and Moore et al,⁵⁹ on the basis of indirect experiments, have suggested that human pluripotent stem cells also lack Ia-like antigenic determinants. Our data are more in accord with recent observations,⁶⁰ which indicate that MCABs directed against HLA-DR (Ia-like) antigens produce marked complement-dependent inhibition of human multipotent (CFU-GEMM) colony formation.

Considerable controversy exists regarding the effects of T lymphocytes on early human erythroid progenitor cell (BFU-E) proliferation *in vitro*. Some investigators have suggested that T cells are required for growth of human peripheral blood BFU-E-derived colonies *in vitro*,¹⁷⁻¹⁹ whereas other studies have demonstrated little dependence of either marrow¹⁹ or peripheral blood²⁰⁻²² BFU-E on T lymphocytes. The explanation for these discrepant findings may relate to methodological differences among laboratories, an intrinsic heterogeneity of human erythroid progenitor cells, different properties and varying maturity of marrow v peripheral blood BFU-E, or inadequate separation and characterization of marrow or peripheral blood subpopulations. Prior investigations have not always accurately assessed the extent of T cell removal or progenitor cell enrichment after target cell depletion, nor provided evidence to gauge functional T lymphocyte activity after cell separation. Our results demonstrate that marrow T cell depletion (to less than 0.05% of the target MNC population) does not alter *in vitro* growth and differentiation of human BFU-E, CFU-GM, and CFU-GEMM. Marrow T cell depletion affected neither colony cytologic composition nor latency of colony development. T cell repletion *in vitro* could not account for the growth and differentiation of

BFU-E obtained from human T cell-depleted marrow. Because the Mo CM used in our studies was derived from a T lymphoblast cell line,⁴⁵ we examined human marrow and peripheral blood BFU-E growth, before and after T cell depletion, in cultures without Mo CM but containing erythropoietin and reduced concentrations of FCS to optimize detection of BPA. T cell and/or monocyte depletion in this setting again failed to affect growth of human marrow BFU-E in vitro, and the addition of either purified marrow monocytes or T cells to autologous nonadherent T cell-depleted marrow target cells had a negligible effect on erythroid progenitor growth. These results are consistent with preliminary observations indicating that potent BPA-producing cells reside within a human marrow non-T, nonmacrophage radioresistant cell population.⁶¹ The normal colony growth seen in T cell-depleted marrow cultures containing human placental-conditioned medium rather than Mo CM suggests that human marrow CFU-GM also do not require T lymphocytes for optimal in vitro growth and differentiation (human trophoblasts appear to be the cellular source of colony-stimulating activity in placental tissue⁶²). Pluripotent progenitors were assayed in vitro using Mo CM; our studies accordingly do not demonstrate CFU-GEMM in vitro growth to be independent of T cell products.

Monocyte but not T cell depletion profoundly suppressed (>90% inhibition) growth of peripheral blood BFU-E. The addition of autologous T cells to T

cell-depleted nonadherent peripheral blood "null" cells produced only a small increment (18% to 30% of expected colony growth) in BFU-E, while the addition of autologous monocytes to peripheral blood "null" target cells restored BFU-E growth to 88% to 110% of expected values. These latter results are in accord with prior investigations^{22,63} and suggest that monocytes are a major source of BPA for peripheral blood but not marrow BFU-E erythropoietin-dependent differentiation. Although T cell depletion had little effect on in vitro growth of human marrow or peripheral blood BFU-E, and the addition of T cells to marrow or blood "null" cells produced only small increments in erythroid progenitor growth, our findings do not preclude the possibility that human T cells may cooperate with other accessory mononuclear cells in regulation of BFU-E proliferation and differentiation, or that human T cell subsets may have important in vivo or in vitro regulatory influences on hematopoietic progenitors.

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