L-Triiodothyronine Augments Erythropoietic Growth Factor Release From Peripheral Blood and Bone Marrow Leukocytes

By Nicholas Dainiak, David Sutter, and Sandra Kreczko

To investigate cellular mechanisms involved in thyroid hormone stimulation of erythropoiesis, we studied the response of erythroid burst-forming unit (BFU-E) proliferation to L-triiodothyronine (L-T3) in a serum-free culture system. When added directly to culture, L-T3 stimulates erythroid burst formation by normal human bone marrow cells. In contrast, granulocyte-macrophage colony formation is unaffected. Enhancement of erythroid burst formation by L-T3 required the presence of nylon wool adherent cells. Furthermore, incubation of CM with an antibody known to react with and absorb BPA from solution removes the inducible mitogen. Biochemical analysis of CM prepared from unexposed and L-T3 pulse–exposed cells indicates that the rate of protein appearance is accelerated by L-T3 in a fashion that immediately precedes growth factor release and that several polypeptides are quantitatively increased. We conclude that unlike erythropoietin, which is mitogenic for progenitor cells directly, L-T3 enhances BFU-E proliferation indirectly by augmenting the release of soluble BPA-like molecules from accessory cells in culture.

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Materials and Methods

Treatment with L-T3. Peripheral blood and bone marrow were collected from 30 healthy, paid volunteers with informed consent and approval of an institutional review board. Samples were mixed with alpha-medium (Gibco 320-2561, Gibco, Grand Island, NY) plus 20 U/mL preservative-free heparin and separated over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Light-density mononuclear cells were removed, mixed with a carboxyl iron suspension (Technicon Instruments Corp, Tarrytown, NY), and separated over Ficoll-Hypaque again. Interface cells that were composed of >95% lymphocytes were split into equal aliquots and suspended to a density of 5 x 10^6/mL in serum-free alpha-medium with or without L-T3. A stock solution of 3,3',5-triiodothyronine (Sigma Chemical Co, St Louis), 10^{-3} mol/L, was prepared in 95% ethanol and added to alpha-medium to obtain final concentrations of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} mol/L. Cells in each aliquot (L-T3 exposed and unexposed) were incubated in 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY) for 15 to 20 min at 37 °C in humidified air, pelleted by centrifugation at 280 g for 20 minutes, and washed three times. Cell viability assessed by trypan blue exclusion and by staining with ethidium bromide and acridine orange was >99%. In some cases, L-T3 was added directly to the bone marrow culture at final concentrations indicated in Table I and the Figure legends.

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Submitted March 25, 1986; accepted June 23, 1986.

Supported in part by National Institutes of Health Grants AM27071 and AM31060 (N.D.).


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0006-4791/86/6806-0016$3.00/0

Blood, Vol 68, No 6 (December), 1986: pp 1289–1297
Four clots are displayed. Similar results were obtained when sheep erythrocyte-rosetting populations (experiments of separated marrow cells. and cultured at densities of 0.5, 1, 2, 3, 4, and 5 x 10⁶/mL, and passed over a 3-mL nylon fiber column.²⁷ Heparin and separated over Ficoll-Hypaque. Mononuclear cells for each donation, and approval of an institutional review board was granted prior to initiation of this study. The cells were placed in 10 mL of well-spiculated bone marrow was aspirated from the posterior iliac crest of 25 healthy, paid donors. Informed consent was obtained before and cultured at 37 °C and 4% CO₂. pelleted, and resuspended sequentially every 15 to 120 minutes for up to 72 hours. The CM was harvested and tested in culture for mitogenic activity, and protein content and composition were determined. Radioimmunofluorescence for thyroxine and triiodothyronine of CM prepared from cells exposed to 10⁻⁴ mol/L L-T₃ for 90 minutes demonstrated undetectable levels of thyroid hormone.

In several experiments, the CM was subjected to centrifugation at 40,000 g for 60 to 120 minutes. The resulting supernatants and plasma membrane-derived vesicle-rich pellets were assayed for protein and BPA contents.

Preparation of CM. Fresh and preincubated, L-T₃ pulse-exposed and unexposed cells were immediately suspended to a density of 5 x 10⁶/mL in tissue culture flasks in alpha-medium with L-glutamine, streptomycin, and penicillin (GIBCO). The cells were incubated at 37 °C and 4% CO₂, pelleted, and resuspended sequentially every 15 to 120 minutes for up to 72 hours. The CM was harvested and tested in culture for mitogenic activity, and protein content and composition were determined. Radioimmunofluorescence for thyroxine and triiodothyronine of CM prepared from cells exposed to 10⁻⁴ mol/L L-T₃ for 90 minutes demonstrated undetectable levels of thyroid hormone.

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Assay and unitage for erythroid mitogens. Approximately 0.5 mL of well-spiculated bone marrow was aspirated from the posterior iliac crest of 25 healthy, paid donors. Informed consent was obtained for each donation, and approval of an institutional review board was granted prior to initiation of this study. The cells were placed in Eagle’s minimal essential medium (MEM) (GIBCO) containing heparin and separated over Ficoll-Hypaque. Mononuclear cells appearing at the interface were separated, washed three times in alpha-medium, and cultured at densities of 0.5, 1, 2, 3, 4, and 5 x 10⁶/mL. In some cases, light-density cells were first resuspended at 5 x 10⁶/mL, and passed over a 3-mL nylon fiber column.²⁷

Morphologically, light-density, nylon-adherent cells were composed primarily of lymphocytes (mean ± SEM, 55% ± 9%) and monocytes (18% ± 3%), while light-density, nylon-nonadherent cells consisted chiefly of lymphocytes (63% ± 11%) and early and mature myeloid elements (14% ± 6%). Fewer than 5% of the nucleated cells in either fraction were benzidine-positive. Fewer than 3% of the nylon-adherent lymphocytes and greater than 95% of the nylon-nonadherent lymphocytes rosetted with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes.

In other experiments, 6 x 10⁶ light-density cells were coated with mouse anti-B4 antibody (100 μg/mL, 30 minutes, 4 °C) obtained from Dr Albert Najman, Faculte de Medecine Saint-Antoine, Paris. The antigen recognized by this antibody is B cell specific and distinct from other known B cell antigens. It is present on both resting and mitogen-stimulated B cells and is increased with B cell activation. It is absent from resting and activated T cells and myeloid cells. Next, purified sheep antimouse IgG (1 mg/mL) was coupled to sheep erythrocytes by the chromium chloride method using equal volumes of washed packed erythrocytes, 0.1% (wt/vol) chromium chloride, and antibody (five minutes, 23 °C). Antibody-coated marrow cells were then incubated with a 2% solution of sheep erythrocytes for five minutes at 4 °C, and the mixture was centrifuged over Ficoll-Hypaque. Cells at the interface (nonadherent) and in the pellet (adherent to erythrocytes from which they were freed by lysis in 0.17 mol/L NH₄Cl) were separated and added to culture. Nonadherent populations consisted of 1% to 2% surface Ig-bearing cells, and adherent populations consisted of 90% to 96% surface Ig-positive cells by immunofluorescence staining. In addition, tests with T cell-depleted human bone marrow were performed with a cytotoxic murine monoclonal anti-T cell antibody prepared by Dr A. Bernard (Institut Gustave Roussy, France). Briefly, 2 x 10⁶ light-density marrow cells in 1.0 mL alpha-medium were incubated with 200 μg/mL antibody plus an equal volume of rabbit complement (Calbiochem-Behring Corp, La Jolla, CA) for 30 minutes at 37 °C. Fewer than 1% of the cells surviving the incubation rosetted with AET-treated sheep erythrocytes.

Serum-free marrow cultures were established in 0.8% methylcellulose or fibrin clots as described previously. They contained Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO), purified human serum albumin, transferrin, ferric chloride, and 0.0, 0.1, 0.5, 1.0, 2.0, or 4.0 U/mL sheep step III erythropoietin (Connaught Laboratories, Toronto), human urinary erythropoietin (70,000 U/mg, a generous gift of Dr Eugene Goldwasser, University of Chicago), or recombinant human erythropoietin (70,000 to 80,000 U/mg, Amgen Biologicals, Thousand Oaks, CA). Test plates contained 10% (vol/vol) L-T₃, CM, or NCTC-109 (Microbiological Associates, Bethesda, MD), unless otherwise indicated. Cultures were maintained for 12 to 14 days at 37 °C and 4% CO₂ and scored for orange bursts (BFU-E-derived colonies) with a grid under an inverted microscope or (in the case of clots) under 100 x magnification. Bursts consisted of ≥50 (and often as many as 500) cells that were benzidine-positive. Cloning efficiency varied from 15 to 60 bursts/10³ light-density cells plated.

Mitogenic activity was determined in CM prepared from preincubated, L-T₃-exposed cells by comparing burst formation in cultures plated with this CM to that with CM prepared from cells preincubated with alpha-medium alone. One unit of BPA is operationally defined as equivalent to 100% or twofold stimulation above burst formation in cultures containing 10% (vol/vol) NCTC-109. The amount of BPA present in each CM was corrected for the volume of CM added to culture (0.2 mL or 0.1 mL for methylcellulose and fibrin clot cultures, respectively) and is expressed per 1-mL volume. Rates of BPA release were adjusted for time of incubation and are expressed as units per milliliter per hour.

Interactions of L-T₃ with other erythroid mitogens. Because several circulating factors are known to augment erythroid progeni-

### Table 1. Stimulation of BFU-E Proliferation by the Addition of L-T₃ Directly to Adherent and Nonadherent Marrow Cell Cultures

<table>
<thead>
<tr>
<th>Marrow Cell Fraction</th>
<th>L-T₃ (10⁻⁴ mol/L)</th>
<th>No. Bursts/10⁶ Cells</th>
</tr>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>–</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Nylon adherent</td>
<td>–</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Nylon nonadherent</td>
<td>–</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Adherent + nonadherent</td>
<td></td>
<td>28 ± 2</td>
</tr>
<tr>
<td>Unseparated</td>
<td>+</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>Nylon adherent</td>
<td>+</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Nylon nonadherent</td>
<td>+</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Adherent + nonadherent</td>
<td></td>
<td>46 ± 7</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>–</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>B₄-negative (nonadherent)</td>
<td></td>
<td>8 ± 1</td>
</tr>
<tr>
<td>B₄-positive (adherent)</td>
<td>–</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Adherent + nonadherent</td>
<td></td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Unseparated</td>
<td>+</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>B₄-negative (nonadherent)</td>
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<td>12 ± 2</td>
</tr>
<tr>
<td>B₄-positive (adherent)</td>
<td>+</td>
<td>not tested</td>
</tr>
<tr>
<td>Adherent + nonadherent</td>
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<td>25 ± 3</td>
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<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
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<tr>
<td>Unseparated</td>
<td>–</td>
<td>19 ± 3</td>
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<tr>
<td>E rosette-negative</td>
<td>–</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Unseparated</td>
<td>+</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>E rosette-negative</td>
<td>+</td>
<td>27 ± 4</td>
</tr>
</tbody>
</table>
tor cell proliferation,15'16 we considered whether one or more serum mitogens other than erythropoietin may serve as potential cofactors for an L-T3 effect. Serum-free cultures of adherent and nonadherent marrow cells obtained after panning with the anti-B4 antibody were established. L-T3 (10⁻¹⁰ mol/L) was added in the presence and absence of platelet-derived growth factor (PDGF) and/or insulin-like growth factor II (IGF-II) at concentrations that will be indicated in Fig 4. Electrophoretically pure porcine PDGF was obtained from Bethesda Research Laboratories (Gaithersburg, MD). This material has an apparent molecular weight (mol wt) of 38,000 on sodium dodecyl sulfate (SDS)–polyacrylamide gels and is biochemically similar to human PDGF.17 Dr M.P. Czech (University of Massachusetts, Worcester) kindly provided IGF-II purified from serum-free medium conditioned by buffalo rat liver cells (BRl-3A).18 Samples were dissolved in 0.005 N HCl and stored at -20 °C until use. A final volume of 100 μL of growth factor or NCTC-109 was added to culture.

Assay for granulocyte-macrophage colony-stimulating activity (GM-CSA). To determine whether L-T3 induced the release of factors that are mitogenic for the granulocyte/macrophage progenitor (CFU-GM), we assayed media conditioned by L-T3–exposed and unexposed cells plus 1% phytohemagglutinin in human peripheral blood culture. Briefly, the method of Pike and Robinson19 was modified by the addition of fivesecond serum substitute20 or 20% fetal calf serum that was adsorbed with activated charcoal to remove thyroid hormones prior to culture.2 Human blood mononuclear cells served as a standard source of GM-CSA.20 Results were compared to cultures containing CM, NCTC-109, or L-T3 (10⁻¹⁰ mol/L) added directly to culture.

Biochemical characterization of L-T3–induced components. CM and medium fractions were analyzed for total protein by the Bio-Rad microassay (Bio-Rad Laboratories, Richmond, CA) and by the method of Lowry et al,21 respectively, using bovine serum albumin (BSA) as the standard.

For gel electrophoresis, 10 to 30 mL of supernatants of CM was extensively dialyzed, lyophilized, resuspended to 50 μL in distilled water, and electrophoresed in one-dimensional 3.5% to 18% SDS–polyacrylamide gels, according to Laemmli.22 Membrane vesicle–containing pellets were resuspended in Na-phosphate and similarly prepared. Gels were stained first with silver and then with Coomassie brilliant blue (CB) according to Dzandu et al.23 Sialoglycoproteins and lipids (yellow Ag-stained bands) and proteins (blue CB-stained bands) were visualized by this method.

Immunologic characterization of L-T3–induced mitogens. To investigate the relatedness of BPA and L-T3–induced mitogens, we employed a polyclonal antimembrane antibody preparation that is known to cross-react with components of CM supernatants and to neutralize BPA in vitro.24 Membrane vesicle–free supernatants (200 μL) were incubated at 25 °C for 45 minutes with 0, 100, or 200 μg of normal rabbit IgG or rabbit antihuman lymphocyte plasma membrane IgG that was purified on diethylaminoethanol cellulose. Antigen–antibody complexes and free IgG were removed by incubation for 30 minutes at 25 °C with an excess amount of a 10% staphylococcal protein A solution and centrifugation at 1,200 g for 60 minutes. Absorbed supernatants were added to culture, and results were compared to those obtained with assays of unabsorbed supernatants.

Statistical analysis. Mean ± SEM bursts appearing in triplicate 2.0-mL methylcellulose cultures and quadruplicate 125-μL fibrin clots were determined. Data sets were compared by the two-sample ranks test of Wilcoxon-White according to Goldstein.24

RESULTS

Burst stimulation by L-T3 and CM. As shown in Fig 1, relative to burst formation in NCTC-109–containing cultures, bursts were increased in cultures supplemented directly with L-T3 (P < .05). The dose-response curve is biphasic, with an optimal final L-T3 concentration of 10⁻¹⁰ mol/L. Stimulation was similar over the full range of the erythropoietin concentration tested, and no bursts formed in the absence of erythropoietin. In contrast, burst enhancement was greatest in cultures with high marrow cell–seeding densities and was absent as the limiting dilution was approached (see Fig 2). Stimulation was absent in cultures of
cells and that L-T3 promotes the release of one or more erythropoietic growth factors from circulating and marrow mononuclear cells.

**Interactions of L-T3, PDGF, and IGF-II.** Since it is possible that the inability of L-T3 to augment burst formation in cultures of B lymphocyte–depleted marrow cells is due to the lack of a necessary cofactor, we examined the effects of L-T3 in cultures containing other serum mitogens. As shown in Fig 4, we observed that L-T3 activity in B cell–containing (unseparated) marrow cultures was additive with that of PDGF and IGF-II. However, L-T3 did not augment BFU-E proliferation in cultures of B cell–depleted bone marrow regardless of whether PDGF and/or IGF-II were also present. The latter finding suggests that neither PDGF nor IGF-II exerts permissive activity for L-T3 action on erythroid burst formation. However, the possibility that other serum molecules may serve as cofactors has not been excluded.

**Kinetics of L-T3–induced growth factor release.** To quantify L-T3 effects on growth factor production, we measured cumulative amounts of BPA and/or BPA-like factors released by cells that were sequentially pelleted and resuspended every 30 to 60 minutes for four hours and after a 24-hour incubation in fresh alpha-medium. The amount of BPA released into CM from L-T3–exposed cells was approximately twice that released from unexposed cells \( (P < .05) \). L-T3 enhancement of cumulative release was maximal at four hours (from 12 ± 3 to 31 ± 3 U/mL for medium conditioned by unexposed and exposed cells, respectively)

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**Fig 3.** Effects of preincubation of leukocytes with L-T3 on growth factor production. Mean ± SEM bursts are shown for methylcellulose cultures containing 10% (vol/vol) NCTC-109 (open circle), CM prepared from L-T3–unexposed cells (zero), and CM prepared from cells pulse exposed to L-T3 90 minutes at the indicated concentrations. Cultures contained 2.0 IU/mL human erythropoietin. Circles. CM prepared from circulating cells; triangles, CM prepared from light-density marrow cells.

**Fig 4.** Influence of PDGF and IGF-II on L-T3 activity. Mean ± SEM bursts/2 x 10^6 cells are shown for cultures of unseparated (A), B4-negative (B), and B4-positive (C) marrow populations obtained after indirect panning (see Materials and Methods). Cultures contained 2.0 IU/mL human erythropoietin plus 10% (vol/vol) NCTC-109, L-T3 (10^-8 mol/L), IGF-II (10 ng/mL), and/or PDGF (10 ng/mL).
and enhancement declined by 24 hours (from 18 ± 3 to 33 ± 2 U/mL, respectively).

Next, we determined the effects of preincubation with L-T3 on rate of growth factor release into CM. As shown in Fig 5, L-T3 transiently accelerates the rate of release of growth-promoting molecules from circulating leukocytes. Peak release (13 ± 2 U/mL/h) occurs at three hours of incubation, and rates of release are similar to control values by 24 hours (7 ± 2 v 8 ± 2 U/mL/h, respectively). Therefore, although the total amount of BPA released from L-T3–exposed cells after 24 hours (and also after 48 and 72 hours, not shown) is enhanced relative to that released from unexposed cells, rates of release are virtually equivalent by 24 hours of incubation.

**L-T3 activity on soluble vesicle-rich fractions.** Since L-T3 might exert a differential effect on the growth-promoting activities associated with each of these physically separable fractions, CM prepared from L-T3–exposed and unexposed leukocytes was fractionated and assayed in culture. Figure 6 shows that approximately half of assayable BPA present in CM is soluble and that L-T3–induced augmentation of growth-promoting activity is fully recovered in the soluble fraction. Moreover, the effects of L-T3 preexposure are most pronounced at suboptimal CM supernatant concentrations (10% and 20%, vol/vol; CM resulted in 3.1- and 2.0-fold stimulations of BPA release relative to unexposed cells, respectively) while a high concentration (30%) of CM showed no difference in BPA release. The data suggest that L-T3 induces the release of cytosolic or soluble membrane–derived molecules that appear to have BPA or BPA-like biologic activity in culture.

**Characterization of L-T3–induced cellular factors.** To characterize effects of L-T3 on the composition of cellular components released during incubation, we measured the total protein contents of CM prepared from exposed and unexposed cells. Both cumulative protein release and rate of protein release were augmented by pulse exposure to L-T3 (Fig 7). Acceleration of the rate of protein release from 23 to 58 μg/mL/h occurred within two hours of incubation and rapidly decreased over time. Cumulative protein release by 24 hours was nearly twofold higher in CM prepared from L-T3–exposed cells (93 v 57 μg/mL). Cell viabilities after 24, 48, and 72 hours of incubation in serum-free media were 99%, 96%, and 87%, respectively. Longer incubations in serum-free medium resulted in excessive cell death (>15%). When analyzed on SDS/polyacrylamide gels, CM supernatant from L-T3–exposed cells contained increased amounts of glycoproteins and proteins with a mol wt approximate of 30 and 70 kilodaltons, respectively, relative to those present in supernatant prepared for unexposed cells. In contrast, no obvious differences were observed in the respective CM pellets (data not shown).

Next, we asked whether erythroid BPA might be related immunologically to the erythroid growth factor(s) induced by L-T3. Antimembrane IgG known to react with and neutralize soluble BPA was used to adsorb growth factors from supernatants of CM prepared from L-T3 pulse-exposed cells. Figure 8 shows that erythroid mitogens were removed from CM supernatant by incubation with IgG, followed by the addition of an excess amount of staphylococcal protein A and removal of the solid phase by centrifugation. In contrast, incubation with staphylococcal protein A alone or normal rabbit IgG plus staphylococcal protein A had no effect on the capacity of CM supernatant to support burst formation (Fig 8). The data raise the possibility that L-T3–induced factors and BPA that is spontaneously released from mononuclear cells may share antigenic determinants.

**Fig 5. Influence of L-T3 on the rate of release of BPA and/or BPA-like molecules.** Mean ± SEM U/mL/h are shown for two studies. CM preparation and marrow culture were performed as described in the legend to Fig 4. Cultures with NCTC-109 alone contained 26 ± 3 bursts/2 x 10^5 cells. Rates of release were calculated by adjusting for the time of incubation. L-T3 markedly augments the rate of BPA released within the first four hours of incubation (P > .10). Open circles, BPA in CM prepared from L-T3–exposed cells; closed circles, in CM prepared from unexposed cells.

**Fig 6. Effects of L-T3 on growth factors expressed by CM fractions.** Whole CM prepared from circulating leukocytes that were preincubated with and without 10^-8 mol/L L-T3 for 90 minutes was separated into supernatants (SUP) and membrane vesicle–containing pellets by high-speed centrifugation. Pellets were resuspended to the starting volume in NCTC-109. Mean ± SEM of bursts are shown for methylcellulose cultures containing 2.0 IU/mL human erythropoietin and 10% (vol/vol) whole CM, CM fractions, or NCTC-109. Cultures with 100% proliferation contained 115 bursts/2 x 10^6 marrow cells. Similar results were obtained in tests of subfractions of bone marrow cell CM.
**Influence of L-T3 on CFU-GM proliferation.** GM-CSA is known to be present in media conditioned by a variety of cell types including circulating light-density cells.\(^{20-27}\) To investigate whether L-T3 augments the production of GM-CSA-like growth factors in a fashion analogous to induction of BPA-like molecule release, we assayed CM prepared from L-T3-exposed and unexposed cells for GM-CSA. Table 2 shows that while GM-CSA is present in our CM preparation, preexposure to L-T3 has no apparent enhancing effect on the expression of this activity in medium conditioned by such cells. Furthermore, addition of L-T3 to serum-free culture likewise does not augment CFU-GM proliferation (Table 2).

**DISCUSSION**

It is well known that clinical hypothyroidism is often complicated by reductions in red cell mass and total blood volume\(^{28,29}\) and by normochromic, normocytic anemia.\(^{30,31}\) The observation that anemia may be corrected by thyroxine alone\(^{32}\) strongly suggests that it may be a consequence of hypothyroidism per se. To explain such in vivo findings, investigations have been undertaken that underscore the importance of thyroid hormones in the maintenance of adequate erythropoietin production\(^{33-35}\) and in the direct stimulation of erythroid progenitor cells.\(^{36-38}\) In this communication, we characterize a hematopoietic target cell population of thyroid hormones by utilizing a biochemically defined human bone marrow culture system while avoiding contamination with unknown quantities of polypeptide hormones, BPA, and extraneous growth factors.\(^{39}\) Since bovine and fetal calf sera contain biologically active L-T3 in concentrations of up to several hundred nanograms per milliliter or \(10^{-8}\) mol/L, assays for L-T3-dependent growth effects are simplified under serum-free conditions.

Several observations suggest that L-T3 augments erythropoiesis in vitro by stimulating local release of paracrine factors such as GM-CSA.\(^{38}\) Consequently, L-T3 may have a role in mediating the anemia in clinical hypothyroidism. The release of GM-CSA-like factors from L-T3-exposed CM is depicted in Fig. 7, A and B. Growth-promoting activity in medium conditioned by such cells was assayed for by measuring CFU-GM proliferation (Table 2).

**Table 2. Effects of L-T3 on CFU-GM Proliferation**

<table>
<thead>
<tr>
<th>Addition to Culture Underlayer</th>
<th>No. Colonies/10⁵ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC-109</td>
<td>154 ± 14</td>
</tr>
<tr>
<td>CM</td>
<td>259 ± 28</td>
</tr>
<tr>
<td>CM + L-T3 (10⁻⁸ mol/L)</td>
<td>265 ± 23</td>
</tr>
<tr>
<td>L-T3 (10⁻⁸ mol/L) — direct addition</td>
<td>145 ± 20</td>
</tr>
<tr>
<td>NCTC-109</td>
<td>127 ± 18</td>
</tr>
<tr>
<td>CM</td>
<td>180 ± 23</td>
</tr>
<tr>
<td>CM + L-T3 (10⁻¹⁰ mol/L)</td>
<td>169 ± 18</td>
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<td>CM + L-T3 (10⁻⁹ mol/L)</td>
<td>185 ± 13</td>
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<tr>
<td>CM + L-T3 (10⁻⁸ mol/L)</td>
<td>178 ± 20</td>
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<tr>
<td>CM + L-T3 (10⁻⁹ mol/L)</td>
<td>189 ± 25</td>
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<td>CM + L-T3 (10⁻⁸ mol/L)</td>
<td>195 ± 19</td>
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<td>L-T3 (10⁻¹⁰ mol/L) — direct addition</td>
<td>115 ± 15</td>
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<td>L-T3 (10⁻⁹ mol/L) — direct addition</td>
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<td>L-T3 (10⁻⁸ mol/L) — direct addition</td>
<td>139 ± 9</td>
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<td>L-T3 (10⁻⁷ mol/L) — direct addition</td>
<td>125 ± 12</td>
</tr>
<tr>
<td>L-T3 (10⁻⁶ mol/L) — direct addition</td>
<td>140 ± 16</td>
</tr>
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</table>

Mean ± SEM of colonies (containing ≥50 cells/clone) plus clusters (containing 3 to 49 cells/clone) are displayed for triplicate determinations.
hormones that, in turn, enhance BFU-E proliferation. First, bone marrow light-density mononuclear cells that are depleted of B lymphocytes by indirect panning with a monoclonal antibody that recognizes most normal B cells in peripheral blood and lymphoid organs or by adherence to nylon wool no longer respond to the mitogenic activity of L-T3 (see Table I). Sensitivity to L-T3 is restored by adding back the depleted cells. On the other hand, T cell lysis has no apparent effect on the ability of L-T3 to augment marrow burst formation. This might be expected since several investigators have shown that unlike their effects on circulating blood and lymphoid organs or by adherence to nylon wool, no longer respond to the mitogenic activity of L-T3. Second, preincubation of BPA-producing peripheral blood and bone marrow cells with L-T3 results in biphasic dose-dependent augmentation of growth factor release that is similar to enhancement observed when L-T3 is added directly to culture (compare dose-response relationships in Fig 1 and 3). It is well known that hormones may show biphasic dose responses wherein growth-promoting effects are absent in vitro at a high-hormone concentration. And third, L-T3 effects were found to be most pronounced at high marrow-seeding densities (Fig 2), suggesting that cellular interactions are important not only for burst formation to take place in vitro but also for the expression of erythropoietic activity by L-T3. Together, the data suggest that L-T3 augment the release of a factor(s) from B lymphocytes or monocytes that stimulates the proliferation of bone marrow BFU-E progenitors. Such a mitogen may act directly on the BFU-E, analogous to the action of T cell– and monocyte-derived BPA on peripheral blood erythroid progenitor cells.

To determine the relatedness of L-T3–inducible proliferation factor(s) and BPA, we made kinetic measurements of the appearance of each during incubation. Both were detectable in CM within four hours from the onset of incubation with the hormone, a finding that is consistent with the release of other mammalian cell growth regulators and lymphokines following short incubation periods. The rapidity of this effect may be explained by activity at a posttranscriptional level of protein synthesis. The rate of L-T3–inducible factor release peaked earlier (three hours vs 24 hours) and declined to a level below that of assayable BPA release by 24 hours, suggesting that L-T3 transiently augments early synthesis and/or exocytosis of lymphocyte mitogens. Peak rates of L-T3–induced protein release immediately preceded peak BPA release (compare data in Fig 5 and 7B). Successful absorption of L-T3–induced mitogens from CM supernatants with antiserum known to bind BPA (Fig 8) is consistent with the hypothesis that inducible burst proliferation factors are antigenically similar to BPA.

A variety of human B lymphocyte plasma membrane components are known to be shed spontaneously into liquid culture medium. We have previously shown that BPA is associated with plasma membrane–derived vesicles present in mitogen-free, liquid culture medium that has been incubated with lymphocytes. Studies with antimembrane antiserum suggest that vesicle-associated BPA and BPA expressed by vesicle-free CM supernatant may share antigenic determinants. To assess whether L-T3 augments the release of vesicle-associated mitogens, activity was assayed in component supernatant and pellet fractions of media conditioned by cells preincubated with L-T3. The results indicate that L-T3 preferentially enhances the release or expression of soluble BPA-like molecules. Furthermore, L-T3 effects can be overwhelmed by adding a saturating concentration of CM to the culture. This suggests that L-T3–induced molecules compete with and/or are functionally similar to BPA. Additional studies employing purified BPA and monoclonal antibodies directed against BPA will be useful in examining this possibility and in probing the relationship of BPA expressed on membrane vesicles to that present in vesicle-free CM supernatants.

Our results are distinct from those obtained with a transformed line of human erythroleukemia (K562) cells that form small colonies of embryonic hemoglobin-containing cells after short-term incubation. Using this assay, Gauwerky and Golde found that 10^-7 mol/L L-T3 induced colony formation in the complete absence of erythropoietin and other differentiation factors. Differences in results are not surprising since normal human bone marrow and transformed leukemic progenitors are known to respond to different chemical inducers (ie, Na butyrate, hemin, etc) and to have different erythropoietin requirements. In addition, the time course of events suggests that such transformed cells are farther along the erythroid differentiation pathway than are the progenitors assayed here. Furthermore, studies with K562 cells were carried out in the presence of approximately 1% serum while those reported here were performed with biochemically defined additives. However, it is known that nuclei of hamster spleen erythroid cells bind radiolabeled thyroid hormone. It is possible that as thyroid hormone receptors appear, direct effects upon more mature nucleated erythroblasts are discerned. This hypothesis is supported by observations of Fuhr and Dunn that globin synthesis is augmented in mouse fetal liver erythroid cells, an effect that is reversible by incubation with erythropoietin. Their data suggest that thyroid hormone acts upon an erythroid cell that is later than the target cell of erythropoietin. To determine whether thyroid hormone also acts directly with specific nuclear receptors on normal human erythroid progenitor cells to stimulate their proliferation, additional studies will require availability of large numbers of highly purified progenitors.

The results of one line of experimentation indicate that L-T3–inducible growth factors are specific for the erythroid lineage. L-T3–inducible growth factors have no apparent activity on granulocyte-macrophage differentiation and/or growth (see Table 2). Whether CFU-megakaryocyte proliferation is altered by thyroid hormones is unknown.
the release of paracrine hormones from accessory leukocytes. Other polypeptide hormones whose target tissues are widespread may express erythropoietic activity by a similar mechanism. Our results emphasize the importance of cellular interactions in regulating normal human erythropoiesis in vitro and possibly in vivo as well. They also point out the complex nature of tissue culture systems that are in widespread use today.

ACKNOWLEDGMENT

We are grateful to Dr Eugene Goldwasser for providing highly purified, human erythropoietin and to Dr Albert Najman for providing monoclonal antibodies.

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