Distinction of Human T-Cell Line (HUT-102)-derived Activity Stimulating Granulocytic Colony Formation in Diffusion Chambers in Vivo from Activities Stimulating Erythroid and Mixed-Colony Formation in Vitro

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ABSTRACT

Medium conditioned in the presence of human HUT-102 T-cell line cells contains activities stimulating human mixed (colony-forming unit, erythroid, granulocyte, macrophage, megakaryocyte) and erythroid (burst-forming unit, erythroid) colony formation in methylcellulose in vitro and granulocyte colony formation in diffusion chambers in mice. The stimulatory effect of HUT-102-conditioned medium on colony-forming unit, granulocyte diffusion chamber was also observed in diffusion chambers implanted in nude mice. The hemopoietic activities were heat stable and could be detected from serum-free conditioned medium. Chromatographically, it was possible to separate colony-forming unit, granulocyte diffusion chamber-stimulating activity from activities stimulating burst-forming unit, erythroid and colony-forming unit, erythroid, granulocyte, macrophage, megakaryocyte. On the other hand, the latter two activities were indistinguishable by the methodology used in this study. Failure to abolish the hemopoietic activities by boiling or by human T-lymphotropic retrovirus type 1 antibody indicates that human T-lymphotropic retrovirus type 1 or its components potentially present in the conditioned medium were not responsible for the stimulatory effects.

INTRODUCTION

Characterization of human and murine hemopoietic precursors which form granulocytic colonies in diffusion chambers in mice in vivo (CFU-DG) and in agar in vitro (CFU-C) has revealed differences suggesting a parent-progeny relationship between the 2 cells (1, 2, 3). Attempts to distinguish between CFU-DG and precursors with a potential to differentiate along erythroid, granulocytic, and megakaryocytic lineages (CFU-S, CFU-MIX) have not been as successful. Densities (4), velocity sedimentation (5), cell cycle status (6, 7), and sensitivity to hypotonic lysis (2, 8) of these precursors are almost identical. Only recovery following cryopreservation (9, 10) or hyperthermia (11) is significantly different. Analysis of single human and murine diffusion chamber colonies by subculture demonstrated the presence of CFU-MIX and 12-day CFU-S, respectively, suggesting that CFU-DG might be at least as primitive as the multipotential precursor cell subpopulations (4, 6, 12).

Most hemopoietic activities stimulating hemopoietic cell prolifera-

1 Supported by NIH Grant R01-AM27423.

2 The abbreviations used are: CFU-DG, colony-forming unit, granulocyte diffusion chamber; CFU-S, colony-forming unit, spleen; CFU-C, colony-forming unit, culture; BFUE, burst-forming unit, erythroid; CFU-MIX, colony-forming unit, erythroid, granulocyte, macrophage, megakaryocyte; CM, conditioned medium; HTLV-1,-2, human T-lymphotropic retrovirus, type 1 or 2; CSA, colony-stimulating activity.

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MATERIALS AND METHODS

Cell Preparation. Bone marrow was aspirated from healthy volunteers after obtaining informed consent and separated with lymphocyte separation medium (by gravity, 1.077 g/ml) (Litton Bionetics, Inc., Kensington, MD). The cells were then suspended in tissue culture medium supplemented with 20% fetal calf serum (Flow Laboratories, Inc., Rockville, MD) and 1% penicillin; streptomycin. In some experiments, bone marrow cells from Institute for Cancer Research mice were utilized.

HUT-102 CM. The T-lymphocyte line HUT-102 was established from a patient with the Sezary syndrome (23, 24). In the presence of lymphocyte CM, lymph node-derived cells were induced to proliferate continuously in RPMI 1640 medium supplemented with 10% fetal calf serum (23) or in serum-free medium consisting of Dulbecco’s phosphate-buffered saline supplemented with insulin and transferrin (25). Full HTLV-1 expression in the cell line was evidenced by reverse transcriptase activity in culture fluid (26) and by presence of type C particles by electron microscopic examination (24). Involvement of HTLV-1 in T-cell transformation has been suggested by its ability to immortalize and alter morphology of T-cells obtained from umbilical cord blood of newborns (27). To determine whether HTLV-1 virus or virus-derived particles were directly responsible for the hemopoietic effects, CM was incubated with HTLV-1 antibody at a dilution 1:40,000 at 37°C for 30 min, a procedure known to neutralize viral particles.

Fractionation of HUT-102 CM by Chromatography. HUT-102 CM was concentrated by ammonium sulphate precipitation (80% saturation), and the precipitate was dissolved in 10 M tris-HCl (pH 7.8) containing 0.1% polyethylene glycol and 0.1 M phenylmethylsulfonyl fluoride. After extensive dialysis against the same buffer, the proteins were chromatographed on a DEAE-Sephadex column using linear 0.7 M NaSO4 gradient for elution. Fractions were assayed for activities stimulating

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colony formation in in vivo and in vitro cultures.

**CFU-DG Assay.** A modification (28, 29) of the technique described by Steinberg et al. (30) was used. Diffusion chambers were filled with 4 x 10^8 human bone marrow cells and implanted in mice which were administered HUT-102 CM i.p. After 14 days of culture, the diffusion chambers were harvested, and the plasma clots attached to Millipore filters were scored as CFU-DG. When nude mice were chosen to host diffusion chambers, surgery was performed in a sterile area, and sterile instruments were used. During the experiment, the cages were protected with bonnets and kept in an isolated room in the vivarium.

**BFU-E Assay.** Cell suspensions (3 x 10^5 cells/plate) to be assayed for BFU-E content were cultured in Petri dishes using a modification (31) of the methylcellulose techniques of Iscove et al. (32). Duplicate plates were incubated at 37°C in a humidified atmosphere of 7.5% CO_2 in air. One unit of human erythropoietin (Toyobo Ltd., Osaka, Japan) and 0.025 to 0.2 ml of CM was added to each plate. On Day 14, aggregates of 40 cells or more were counted as BFU-E.

**CFU-MIX Assay.** A technique (20) modified by Ash et al. (33) was used to assay CFU-MIX. Cells (2 x 10^5/plate) were cultured in 35-mm Petri dishes with 0.025 to 0.2 ml of CM from the HUT-102 cell line, 1 unit of human erythropoietin (Toyobo, Ltd.), 30% fetal calf serum, Iscove’s modified Dulbecco’s medium, 5 x 10^-6 M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 0.9% methylcellulose as a viscous support. After 14 days of incubation at 37°C in humid atmosphere containing 5% CO_2, cultures were examined for the presence of mixed colonies. Mixed colonies were identified by their composition of translucent and hemoglobinized cells. Heterogeneous morphology was verified on slides prepared from individual colonies and stained with Wright’s stain.

**Statistical Analysis.** Ps were calculated with Wilcoxon’s nonparametric rank test.

**RESULTS**

Administration of HUT-102 T-cell CM to mice carrying i.p.-implanted diffusion chambers filled with human marrow cells resulted in a dose-dependent increase in colony formation (Table 1). Stimulation of CFU-DG was also observed after treatment with boiled CM and CM from serum-free HUT-102 cultures (Table 1). When murine marrow served as a target, the number of CFU-DG remained unchanged (data not shown), suggesting species specificity for the stimulatory activity present in HUT-102 CM. The effect of HUT-102 CM on human CFU-DG could also be demonstrated in diffusion chamber cultures implanted in nu/nu mice (Table 1).

Mixed-colony formation in methylcellulose was observed only in cultures containing HUT-102 CM (Table 2). Addition of CM in excess of 0.1 ml did not further increase CFU-MIX numbers. Addition of serum-free CM and CM boiled for 60 min also stimulated CFU-MIX formation (Table 2). Morphological analysis demonstrated a predominance of erythroid and granulocytic elements, although occasionally macrophages and megakaryocytes were seen. The 3 types of HUT-102 CM used also stimulated BFU-E (Table 2). Again, addition of CM to cultures in volumes exceeding 0.1 ml did not result in further enhancement of burst formation.

To determine the relationship between CFU-DG-, BFU-E-, and CFU-MIX-stimulating activity, HUT-102 CM was subjected to DEAE-Sepharose chromatography, and the resulting fractions were assayed in both in vivo and in vitro cultures. As shown in Chart 1, BFU-E-stimulating activity could be separated from CFU-DG-stimulating activity but not from activity stimulating CFU-MIX. Separation of control medium (RPMI 1640 plus 10% fetal calf serum) with chromatography did not yield any fractions with increased BFU-E-, CFU-DG-, or CFU-MIX-stimulating activity levels.

**Table 1**

<table>
<thead>
<tr>
<th>Volume of CM administered to host mice (ml)</th>
<th>CFU-DG/chamber, mean ± SE°F</th>
<th>CFU-DG/chamber, mean ± SE°F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Institute for Cancer Research mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8 ± 2</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>0.5</td>
<td>24 ± 3</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>1.0</td>
<td>53 ± 6</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>1.5</td>
<td>84 ± 8</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>2.0</td>
<td>90 ± 11</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>2.0 (serum-free CM)</td>
<td>83 ± 12</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>2.0 (boiled 60 min)</td>
<td>85 ± 7</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>nu/nu mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26 ± 3</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>2.0</td>
<td>113 ± 11</td>
<td>26 ± 2</td>
</tr>
</tbody>
</table>

*In doses of 0.25 ml/day up to the total dose.

**Table 2**

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>BFU-E/4 x 10^5 bone marrow cells, mean ± SE†</th>
<th>CFU-MIX/2 x 10^5 bone marrow cells, mean ± SE†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>HUT-102 CM</td>
<td>120 ± 5</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>HUT-102 CM (serum free)</td>
<td>90 ± 5</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>HUT-102 CM (boiled 60 min)</td>
<td>96 ± 6</td>
<td>26 ± 2</td>
</tr>
</tbody>
</table>

*Cultures contained 0.1 ml of CM.

†Each point represents 9 observations.
HUMAN T-CELL-DERIVED HEMOPOIETIC ACTIVITIES

els. Neutralization of HTLV-1 particles potentially present in HUT-102 CM medium by HTLV-1 antibody did not abolish its hemopoietic activities.

DISCUSSION

Results from this study indicate that in addition to CSA (34), HUT-102 CM contains factors which stimulate CFU-DG (Table 1) CFU-MIX, and BFU-E (Table 2). Following ion-exchange chromatography, it was determined that CFU-DG-stimulating activity could be separated from BFU-E- and CFU-MIX-stimulating activity (Chart 1), suggesting that the in vivo diffusion chamber culture system detects unique hemopoietic modulators. This is also supported by a previous study (22) in which it was demonstrated that following chromatography, CSA- and CFU-DG-stimulating activity peaked in different fractions and had different heat stabilities. Presence of stimulatory activities in serum-free medium (Tables 1 and 2) indicates that they were not derived from fetal calf serum. Administration of fractionated RPMI 1640 medium supplemented with fetal calf serum did not enhance colony formation, excluding the possibility that the stimulatory activity was present in the medium but masked by a neutralizing inhibitor.

The responsiveness to different humoral stimulators of hemopoietic precursors CFU-C, CFU-DG, and CFU-MIX (Ref. 22; Tables 1 and 2), which all gave rise to granulocytic elements, suggests that at least in culture granulopoiesis can be modulated by several mechanisms. Whether this interpretation is relevant to regulation of granulocyte production in vivo is presently unknown. Involvement of CSA has been suggested by studies (35–41) in which granulocytosis was observed in many patients with a tumor-producing CSA. However, the possibility that factors other than CSA cause granulocytosis was not excluded. This concern is justified by a recent report (42) describing poor correlation between levels of CSA derived from 2 mammary adenocarcinoma tumor lines and granulocyte levels in host mice. The growth of human marrow in diffusion chambers in untreated mice is poor. Therefore, the hosts, prior to chamber implantation, receive irradiation or cyclophosphamide to support colony formation. Whether or not immune suppression of the xenogeneic milieu achieved with these agents is responsible for enhanced growth is unclear. The possibility that HUT-102 CM line-derived factors might block the action of host T-cell factors prompted us to culture human marrow in diffusion chambers in nu/nu mice. Although somewhat improved CFU-DG formation in untreated nu/nu mice in comparison to untreated nude mice was observed (Ref. 29; Table 1), substantial enhancement of CFU-DG was achieved by administration of HUT-102 CM to nu/nu hosts, suggesting that host T-cells do not play a role in colony formation in diffusion chambers in mice.

Since the HUT-102 T-cell line produces HTLV-1 viruses, it was possible that either live viruses or molecular viral constituents were responsible for enhancement of hemopoietic colony formation. This explanation is unlikely because incubation of CM with polyclonal HTLV-1 antibody did not abrogate the stimulation. Also, direct effect on hemopoietic precursors by viruses can be confidently ignored because even after boiling, which kills viruses (43), the CM stimulated CFU-DG, BFU-E, and CFU-MIX (Tables 1 and 2). These observations do not exclude a role for retroviruses in the production of hemopoietic activities by lymphocytes. The contrary is probably true, because several cell lines producing hemopoietic activities have been developed by transforming normal lymphocytes using HTLV-1 and HTLV-2 (44).

We conclude that the HUT-102 T-cell line produces CFU-DG-stimulating activity which can be separated from activities stimulating BFU-E and CFU-MIX. Whether it is identical with the CFU-DG-stimulating activity elaborated by the Mo T-cell line will be possibly determined following biochemical characterization and generation of antibodies.

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