PRACTICAL IN VITRO ASSAY SYSTEMS FOR THE MEASUREMENT OF HEMATOPOIETIC GROWTH FACTORS

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SUMMARY: A tetrazolium salt (MTT) was used to develop rapid, practical colorimetric assays for the measurement and differential identification of picomolar levels of certain hematopoietic growth factors (GM-CSF, CSF-1, and Multi-CSF/IL-3). The signal generated is directly proportional to the number of viable target cells present after 2 d of culture. Thus, this method can be used to quantitate proliferation or survival of all types of factor responsive cells in vitro. Assays using MTT reduction or [^{3}H]thymidine as read-outs give comparable results; however, MTT reduction is easier, faster, and less expensive. The use of two target cell lines, each with well-characterized growth factor responses, and with differing responsiveness to the myeloid growth factors CSF-1, GM-CSF, and Multi-CSF/IL-3, is described. We further detail steps necessary to adapt other target cells to this convenient assay system.

Key words: colorimetric assay, hematopoietic growth factors, lymphokine assays, MTT, proliferation assays

I. INTRODUCTION

In 1961, Till and McCulloch (1) observed that pluripotential stem cells (CFU-s) give rise to spleen colonies after transplantation of hematopoietic cells into irradiated recipients, opening the door for in vitro investigation of both multipotential and unipotential hematopoietic cell growth. Within a few years, Pluznik and Sachs (2) and Bradley and Metcalf (3) introduced the first in vitro techniques for clonal cell culture, utilizing methylcellulose or semisolid agar conditions to localize growing colonies of progenitor cells. These methods led to the development of assay systems for precursor cells of the various hematopoietic lineages (4–6). Different precursor types are defined by their ability to form clones of morphologically recognizable mature blood cells. The frequency of a specific hematopoietic cell type within a population, as well as the relative concentration of specific growth factor necessary for its proliferation and differentiation, can be determined by counting colonies of specific morphology.

These "clonogenic" in vitro assay systems have been useful in studying the response of committed progenitor cells to specific growth factors (7,8). These include the granulocyte-macrophage colony-forming cells (GM-CFC), responsive to G-CSF, CSF-1, GM-CSF, and Multi-CSF/IL-3; the burst-forming unit-erythroid (BFU-E), for early erythroid cells, responsive to erythropoietin and Multi-CSF/IL-3; the colony-forming unit-erythroid (CFU-E), for late erythroid cells, responsive to erythopoietin; as well as assays for progenitors of megakaryocytes, eosinophils, basophils, and T or B lymphocytes [reviewed by Metcalf (4)].

All of the above hematopoietic growth factors (HGF) have been isolated, purified, molecularly cloned, and shown to bind to different receptors on target cells [reviewed in (9,10)]. In this paper, we focus on the assay of major subgroups of biochemically distinct glycoprotein HGF that, in the mouse, interact to control the production and function of granulocytes and macrophages (Table 1). Three of these factors (CSF-1, GM-CSF, and G-CSF) have also been identified and studied in the human system. Recently, human Multi-CSF/IL-3 has been cloned and shown to have similar properties as murine multi-CSF (12).

Although the classical clonogenic assays remain the standard for the qualitative study of HGF, these methods are indirect, tedious, and slow because the effects of HGF on progenitor cells are detected only by *visual* observation of the results of their proliferation and differentiation [*see* (7,11) for details on carrying out clonogenic assays]. Thus, a more quantitative assay system was needed to better study the effects of HGF on cell proliferation. The advent of cell lines, dependent on HGF for growth, has led to development of culture assays that quantitate mitogenic stimulation using the number of cells incorporating radioactive tritiated thymidine ([^{3}H]TdR) into DNA; a measure of cells cycling through S phase (13). Unfortunately, radioactive methods are expensive, requiring special equipment (scintillation or gamma counters) and mate-

TABLE 1

MYELOID SUBSETS OF MURINE AND HUMAN GRANULOCYTE-MACROPHAGE HEMATOPOIETIC GROWTH FACTORS

Name	Most Common Abbreviation(s)	Molecular Weight	Cloned?	Comment
Granulocyte-macrophage colony-stimulating factor	GM-CSF	23 000 dalton	yes	murine does not crossreact with human and human does not crossreact with murine
Granulocyte-colony-stimulating factor	G-CSF	25 000 dalton	yes	murine and human are reciprocally crossreactive
Multipotential colony-stimulating factor	Multi-CSF IL-3	23 000 to 28 000 dalton	yes	murine and human crossreactivity not yet thoroughly studied
Macrophage colony-stimulating factor	CSF-1 M-CSF	70 000 dalton	yes	murine does not crossreact with human but human crossreacts with murine

rials (scintillation fluid and vials) and have a certain risk associated with the use of radioactive compounds.

In 1983, Mosmann (14) described an in vitro colorimetric method for measuring the cellular proliferative response, which uses the dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], abbreviated MTT. Tetrazolium salts are reduced to an insoluble blue crystal in active mitochondria, allowing for a colorimetric reaction to occur only in viable cells. Thus, the extent of reduction in culture is proportional to the number of metabolically active cells and is ideal for quantification of changes in cell number that result from stimulation of growth or survival. The method is rapid, does not require special equipment (although it is best read on a scanning multiwell spectrophotometer), can be made quantitative, and obviates the use of radioactive compounds; however, MTT is considered a carcinogen and needs to be handled with care.

We describe an experimental approach for developing practical, rapid in vitro assays for quantitating hematopoietic growth factors. Our approach is general, which should apply to any target cell for any HGF.

II. MATERIALS

A. Equipment

Freezer, biological, to –85° C, no. 7110 Queue Cryostar[1]

CO_2 Incubator, biological, 37° C, 5% CO_2, no. 3326 S/N 33283-197, Forma Scientific[2]

Scanning multiwell spectrophotometer, Microplate ELISA Reader, no. MR600, Dynatech[3]

Sterile fume hood, no. BH-2001B, Western Scientific[4]

Shaker, IKA-Vibrax-VXR, no. 142064, Janke & Kunkel[5]

Scintillation counter, no. C4450, Hewlett-Packard[6]

Scintillation vials, No. 600-192 Hewlett-Packard[6]

Cell Harvestor, no. 7810400, Flow Laboratories[7]

Pipettes, analytical

Single-channel, adjustable 5–50 μl, no. 821 Socorex[8]

Single-channel, adjustable 50–100 μl, no. 821[8]

Multichannel, 8–12 place, 25 μl Titertek Lab Systems[9]

Multichannel, 8–12 place, 50 μl Titertek[9]

Multichannel, 8–12 place, 100 μl Titertek[9]

Repeater, 10–20 μl, no. 4780 Eppendorf, Brinkman Instruments[10]

Pipette tips, no. 22–34 160-0 Eppendorf[10]

Hemacytometer, no. 02-671-5, Bright Line, Fisher Scientific[11]

B. Glassware

Pipettes, serological, 1-ml, no. 52961-075; 5 ml, no. 52961-111; 10 ml, no. 52961-133[4]

Pipettes, Pasteur, 9 in. disposable, no. 13-678-6H[11]

Centrifuge tubes, conical, polystyrene, sterile, 15 ml, no. C3973-15; 50 ml, no. C3973-50, Corning, CANLAB[12]

Tissue culture flasks, polystyrene, sterile, T-25, no. T-4160-25; T-75, no. T-4160-75, Corning[12]

Petri dishes, disposable, sterile, plastic, 100 × 15 mm, no. 08-757-13[11]

Microtiter plates, 96-well, flat-bottom, no. 76-003-05 and no. 76-035-05, Linbro[7]

C. Chemicals and reagents

Iscove's modified Dulbecco's medium (IMDM), no. 430-020 GIBCO[13]

Fetal bovine serum (FBS), sterile, mycoplasma and virus-free, no. 29-101-54, Lot 29101065[7]

Phosphate buffered saline (PBS), pH 7.3, prepared ourselves from reagent grade chemicals

Isopropanol (2-propanol), reagent grade, no. 8600-1, Caledon[14]

HCl, concentrated, no. 9530-1, Baker[15]

EDTA, no. CAS-6381-92-6[11]

MTT, store at 0–4° C, avoid contact with light, no. M-2128, Sigma[16]

[^{3}H]TdR, specific activity = 20.0 Ci/mmol, no. NET-027X Du Pont, New England Nuclear[17]

Scintillation cocktail, Omnifluor, no. NEF-906[17]

Triton X-100, no. T-6878[16]

Gentamicin sulfate, USP, no. 600-5750[13]

Lipopolysaccharide (LPS), from *E. coli*, no. L-3254[16]

4-Phorbol 12-myristate 13-acetate (PMA), no. P-8139[16]

Horse serum, No. 230-6050, Lot 20K0140[13]

Human serum, normal, group AB, pooled, Canadian Red Cross[18]

Rabbit serum, normal

Concanavalin A (Con A), lot EL 13835, Pharmacia Fine Chemicals[19]

Phytohemagglutinin (PHA), lot K835620, Wellcome Research Laboratories[20]

Pokeweed, lot L-9379[16]

D. Cells

DA1.2, Cloned DA-1 cell line, D. R. Branch, University of Alberta

5/10.14, Subclone of 5/10 cells, D. R. Branch, University of Alberta

WEHI-3, murine myelomonocytic cell line, American Type Culture Collection (ATCC)[21]

L-cells, murine, clone L929 (ATCC)[21]

5637, human bladder carcinoma cell line[21]

MiaPaCa, human pancreatic cancer cell line[21]

E. Hematopoietic growth factors

WEHI-3 conditioned medium (WEHI-3 CM), supernatant from WEHI-3 cells, cultured using IMDM + 15% FBS + 10^{-5} *M* 2-mercaptoethanol. Supernatant was collected when cells were slightly past log-phase growth (medium was orange-yellow). WEHI-3 CM contains multi-CSF/IL-3 and CSF-1 (15,16).

L-cell conditioned medium, supernatant from murine L-cells cultured in IMDM + 10% FBS. L-cell conditioned medium contains CSF-1 (17).

CSF-1, murine, obtained from L-cell conditioned medium after precipatation using Ca phosphate as previously described (18).

CSF-1, human, obtained from MiaPaCa cell-conditioned medium after precipitation using Ca phosphate as previously described for murine CSF-1 from L-cell conditioned medium (18).

rGM-CSF, recombinant murine GM-CSF from COS cells, a gift from Dr. R. J. Tushinski, Immunex[22]

Multi-CSF/IL-3, murine, purified, a gift from Dr. J. N. Ihle[23]

rmulti-CSF, recombinant murine multi-CSF from yeast cells, a gift from Dr. T. Mosmann[24]

IL-2, human, purified, a gift from Dr. P. Mannoni[25]

rIFN2, recombinant human gamma interferon from *E. coli*, a gift from Biogen[26]

rEPO, recombinant human erythropoietin from Chinese hamster ovary cells, a gift from Dr. E. Fritsch[27]

EPO, murine, purified, lot 10405, HyClone[28]

5637 Conditioned medium, supernatant from 5637 cell line. Conditioned medium contains the following human hematopoietic growth factors: GM-CSF, G-CSF, hemopoietin 1 (H1), pluripoietin alpha, and leukemia blast growth factor (19–22).

III. PROCEDURE

A. Target cell lines

DA-1 is a nonadherent murine cell line, originally isolated from a Moloney leukemia virus-induced lymphoma, reported by Dr. J. N. Ihle in 1985 (23). The morphology of the DA-1 cell line is lymphoblastic with no detectable granulation. A rapidly growing clone, DA1.2, was isolated in our laboratory from methylcellulose cultures containing WEHI-3-conditioned medium. This clone was selected for growth factor assays because of its greater sensitivity to both Multi-CSF and GM-CSF compared to the parent DA-1 line. The DA1.2 clone is maintained in suspension culture in T-25 or T-75 tissue culture flasks using IMDM supplemented with 20% WEHI-3-conditioned medium and 15% FBS.

The 5/10 murine macrophage cell line was isolated in our laboratory by limiting dilution of MM-5.47 cells in medium containing both CSF-1 and Multi-CSF. MM-5.47 cells derived from a rapidly growing clone of C3H/HeJ monoblasts [bone marrow derived macrophages at Day 3 of culture (24)] which had been infected with a murine retroviral vector containing the v-*myc* gene from avian OK10 virus (24). The cells are morphologically macrophages, strongly phagocytic and adherent, and Mac-1 +. The subclone 5/10.14 was isolated from 5/10 cells by limiting dilution in the presence of high concentrations of CSF-1, and was selected for its responsiveness to and absolute requirement for CSF-1, Multi-CSF, or GM-CSF. The 5/10.14 cells are maintained in continuous culture in petri dishes using IMDM supplemented with 20% L-cell-conditioned medium and 15% FBS and passaged at 5×10^5 cells/ml as described below for harvesting.

Both DA1.2 and 5/10.14 cell lines were tested and were free of microorganisms, including *Mycoplasma sp.*

B. DA1.2 assay

Known HGF or unknown samples were serially diluted (doubling dilutions) in IMDM supplemented with 5% FBS and gentamicin (1 μl/ml), using a 96-well, flat-bottom microtiter plate (Linbro, no. 76-003-05), to give a final volume of 25 μl/well. A multichannel pipette (25 μl) was used and pipette tips were rinsed with medium between each dilution. Negative controls consisted of diluent alone. Log-phase growing DA1.2 cells were selected for each assay procedure. Cells were washed four times with sterile PBS, the cell number adjusted to 4×10^5 cells/ml in IMDM containing 5% FBS, and 25 μl added to all the wells of the microtiter plate (10^4 cells/well). The final well volume was 50 μl/well. Plates were incubated at 37° C in an atmosphere of 5% CO_2, pH 7.35. At 48 h, proliferation was measured by the colorimetric MTT assay. Assays were performed in triplicate or quadruplicate and results expressed as the mean ± SD.

C. 5/10.14 Assay

Known HGF or unknown samples were serially diluted (doubling dilutions) in IMDM supplemented with 15% FBS plus gentamicin (1 μl/ml), using a 96-well, flat-bottom microtiter plate (Linbro, no. 76-003-05 or 76-035-05), to give a final volume of 50 μl/well. A multichannel pipette (50 μl) was used and pipette tips were rinsed with medium between each dilution. Negative controls consisted of diluent alone. Adherent 5/10.14 cells were harvested from petri dishes after treatment with 5 ml sterile, cold EDTA solution (2 m*M* EDTA diluted in PBS) for 5 min at 4° C. The harvested cells were washed four times with sterile PBS, the cell number adjusted to 2×10^4 cells/ml in IMDM containing 15% FBS, and 50 μl added to all wells of the microtiter plate (10^3 cells/well). The final well volume was 100 μl/well. Plates were incubated at 37° C in an atmosphere of 5% CO_2. At 48 h, proliferation was measured by the colorimetric MTT assay or by [^{3}H]TdR uptake. Assays were run in triplicate or quadruplicate and the results expressed as the mean ± SD.

D. Colorimetric MTT assay

MTT is dissolved in distilled water (5 mg/ml) and centrifuged to remove any remaining insoluble residue. This solution is stored in the dark, at 4° C, and is stable for at least 1 mo. MTT solution is added directly to each microtiter well in the DA1.2 assay using a repeater pipette (set to deliver 10 μl). In the 5/10.14 assay, the microtiter wells are first decanted completely by inversion and then 50 μl of MTT solution (1 part MTT solution diluted in 9 parts IMDM supplemented with 1% FBS) is added to all wells of the assay using a multichannel pipette (50 μl). After addition of MTT solution, the plates are incubated at 37° C for 4 to 5 h. After incubation, acid-isopropanol (100 μl of 0.04 *N* HCl in isopropanol; prepared by adding 0.333 ml of concentrated HCl to 100 ml of isopropanol) is added to all wells and mixed thoroughly on a shaker (moderately high setting) for 5 to 10 min until all blue crystals are dissolved. The plates are read on a Dynatech MR600 Microplate reader with the wavelength select switch in the λT/λR position, and using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.00. Plates should be read as soon as possible after addition of acid-isopropanol.

E. [^{3}H]TdR assay

[^{3}H]TdR solution (1 μCi; spec. act. of 20.0 Ci/mmol, diluted 1:19 in IMDM, 20 μl = 1 μCi) is added directly to the microtiter wells using a repeater pipette (set to deliver 20 μl), and the plates incubated at 37 °C for 4 h. After incubation, DA1.2 cells, which are nonadherent, are harvested onto absorbant glass-fiber paper using a cell harvester. In contrast, 5/10.14 cells, because they are strongly adherent, required the addition of 100 μl of 0.1% Triton X-100 (diluted in PBS) followed by gentle mixing on a shaker for 2 min before harvesting. Absorbant paper containing harvested cells was dried for 30 min in a drying oven or air dried overnight. Circles containing cells were cut out of the paper and put into appropriate scintillation vials with 1 ml of scintillation solution, and the radioactivity of each vial measured using a scintillation counter.

F. Optimal conditions for growth factor response

Conditions for hematopoietic growth factor assays must be such that the cell density and length of incubation time allow cells to proliferate without the constraints of nutrient depletion. In practice this means that a constant and maximally stimulating level of HGF should elicit a response directly proportional to cell number, i.e., plots of response vs. cell number should be linear and intersect the origin (*see* Fig. 3). Using this approach, the optimal conditions for an MTT or [^{3}H]TdR assay that allow for a linear response with the highest signal-to-noise ratio can be accurately determined.

Using DA1.2 cells, the cell density was varied over the range 500 to 20000 cells/microtiter well. A fixed, high concentration of hematopoietic growth factor (12.5% to 50% WEHI-3 CM) was used to maximally stimulate the cells, and the response over the cell range analyzed. Both length of incubation (usually 1 to 4 d) and optimal cell number were variables to be determined for a given assay. Using an MTT assay, optimal conditions were found to be incubation for 48 h using 1×10^4 cells/well (Fig. 1 *a*).

Using 5/10.14 cells and a fixed, high concentration of CSF-1, the optimal conditions (a linear response with the highest signal-to-noise ratio by MTT assay) was determined to be incubation for 48 h using 1×10^3 cells/well (Fig. 1 *b*). These conditions were also optimal when using a [^{3}H]TdR assay (Fig. 1 *c*).

G. Dose-response curves

The next step in setting up bioassays for measuring response to hematopoietic growth factors is obtaining relatively pure or recombinant HGF to be used as standards for the assessment of the growth characteristics of a given target cell. Assays need to be performed by titrating, using doubling dilution, each pure HGF against each target cell for activity, using the previously determined optimal growth conditions (*see* Section IIIF). Thus, for each HGF, a characteristic pattern of responsiveness, the so-called dose-response curve, can be determined for a given target cell. The first two dilutions should be chosen so that the response will be maximal. Dilutions are usually carried out to 16 doublings (2^{16}) and the resulting dilutional dose-response curve expressed as the optical density (O.D.), or counts per minute (cpm), as a function of the $\log_2$ of each dilution. Figure 2 *a* indicates the dose-response curves, using MTT, obtained for WEHI-3-conditioned medium, rMulti-CSF, and rGM-CSF using DA1.2 cells. Similar results for Multi-CSF were obtained when using a highly puri-

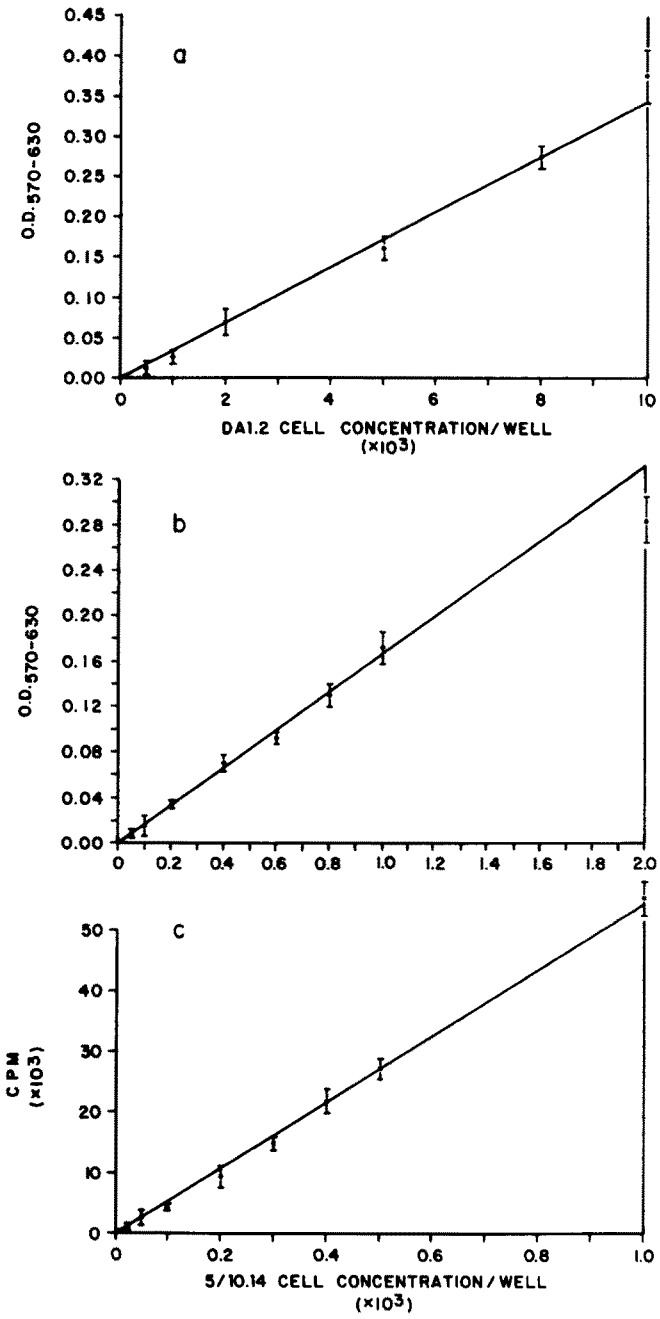

FIG. 1. Response of MTT and [³H]TdR assays as a function of cell number at a fixed level of hematopoietic growth factor. *a*, DA1.2 cells were cultured at 500 to 10000 cells/well in 50 μl medium (IMDM + 5% FBS + 12.5% WEHI-3 CM) in 96-well, flat-bottomed plates. After 48 h, MTT (10 μl of 5 mg/ml stock) was added to all wells and the plates incubated at 37° C for 5 h, developed, and measured. Each point shows the mean and standard deviation of four replicates. The *straight line* plotted is the best fit line calculated using linear regression analysis (r^2 = 0.995). *b*, 5/10.14 cells were cultured at 50 to 2000 cells/well in 100 μl medium (IMDM + 15% FBS + 6.25% CSF-1) in 96-well, flat-bottomed plates. After 48 h the supernatant was discarded and MTT (50 μl of 10% stock solution in IMDM) was added to all wells and the plates incubated at 37° C for 5 h, developed, and measured. Each point shows the mean and standard deviation of four replicates. The *straight line* plotted is the best fit calculated by linear regression analysis (r^2 = 0.997) using points from 50 to 1000 cells/well. *c*, 5/10.14 cells were cultured at 25 to 1000 cells/well in 100 μl medium (IMDM + 15% FBS + 6.25% CSF-1) in 96-well, flat-bottomed plates. After 48 h [³H]TdR (1 μCi/well) was added to all wells and the plate incubated at 37° C for 4 h. The cells were treated with 100 μl 0.1% Triton X-100 and harvested onto absorbant paper and counted for radioactivity using a scintillation counter. Each point shows the mean and standard deviation of four replicates. The *straight line* plotted is the best fit calculated using linear regression analysis (r^2 = 0.997).

fied source of natural murine Multi-CSF (data not shown). Figure 2 *b* indicates typical dose-response curves, using MTT, obtained for CSF-1, rMulti-CSF, and rGM-CSF using 5/10.14 cells. Figure 2 *c* shows the dose-response curves obtained by [³H]TdR assay for CSF-1, rMulti-CSF, and rGM-CSF using 5/10.14 cells.

H. Comparison of MTT to [³H]TdR

It is apparent from the linear response curves (Fig. 1 *b*, *c*) and the dose-response curves (Fig. 2 *b*, *c*), that the MTT assay system for measuring response to granulocyte-macrophage hematopoietic growth factors is comparable to the more traditional assays for measuring cell proliferation using [³H]TdR. These results confirm previous data for lymphocyte proliferative responses to the mitogens Con A and LPS (14), and indicate that the colorimetric MTT assay is a suitable method for the measurement of cellular proliferation in vitro.

I. Specificity

It is important to characterize growth responses of a given cell line as thoroughly as possible. Table 2 contains results of the responsiveness of either DA1.2 or 5/10.14 cells to various growth factors, mitogens, and sera. In addition, the DA-1 parent cell line can proliferate in response to purified, natural murine B-cell stimulatory factor (BSF-1), also termed IL-4 (personal communication, P. M. Flood, Howard Hughes Medical Center Institute, Yale University, New Haven, CT).

J. Quantitation

The activity of any HGF in a sample can be quantified (see legend, Fig. 3) provided the following criteria are met: (a) a standard for the HGF is available for quantitative comparison (*see* below); (b) the target cells used in the assay detect only this HGF (i.e., they respond to only one in a mixture of HGF or there is only one HGF present in the sample); and (c) the sample should not inhibit growth of the target cells. If all three provisions hold, samples will generate a family of sigmoidal dose-response curves of identical shape and heights (e.g., Fig. 3 for CSF-1 samples). Thus, dose-response curves for samples should not vary by more than 20% from the standard in either maximal (plateau) response or slope of the tangent at the inflection (*straight lines* in Fig. 3). Lack of correspondence indicates that one or more of the above provisions does not hold and that the sample cannot be appropriately quantitated. Also note that both differential characterization and quantitation of HGF

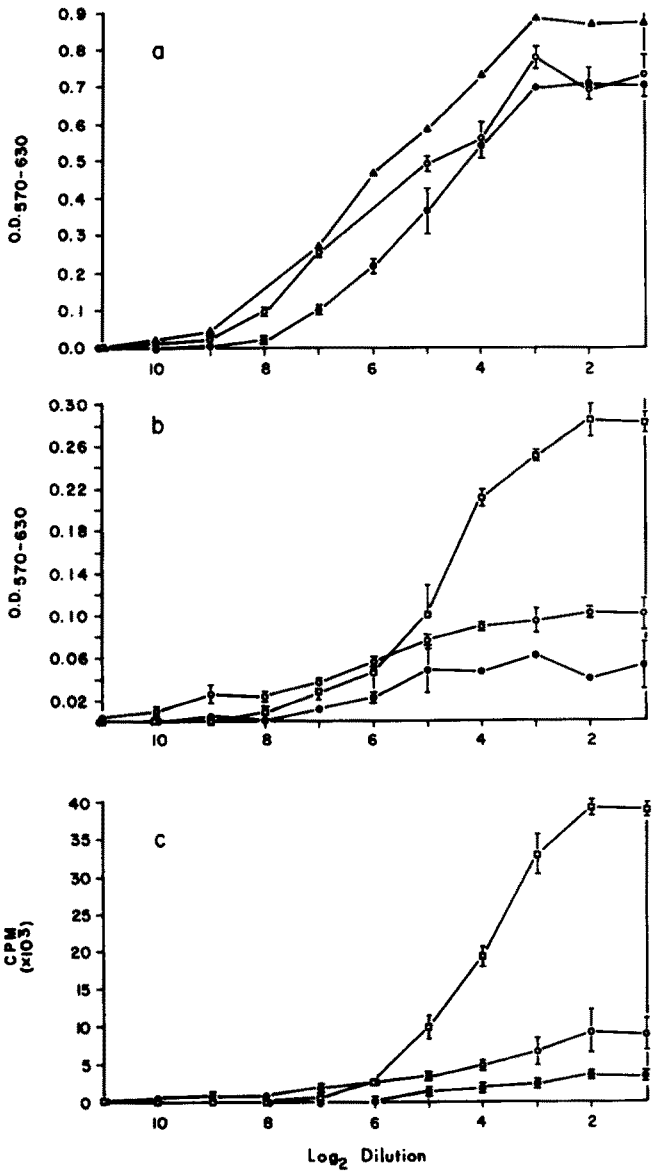

FIG. 2. Dose-response curves of selected hematopoietic growth factors monitored by MTT reduction or [^{3}H]TdR uptake under optimal growth conditions (*see* Fig. 1). The means and standard deviations of three replicates per point are shown, except when using WEHI-3 CM, where only single data points were plotted. *a*, DA1.2 response, using MTT reduction, to serial doubling dilutions of WEHI-3 CM = ▲; rGM-CSF = ●; and rMulti-CSF = ○. *b*, 5/10.14 response, using MTT reduction to serial doubling dilutions of CSF-1 = □; rGM-CSF = ○; and rMulti-CSF = ●. *c*, 5/10.14 response, using [^{3}H]-TdR uptake, to serial doubling dilutions of CSF-1 = □; rGM-CSF = ○; and rMulti-CSF = ●.

require several target cell lines of defined growth factor responsiveness.

IV. DISCUSSION

The use of a colorimetric MTT assay provides a rapid method for the study of cell proliferation. Our method is slightly modified from the original report by Mosmann (14). In preliminary experiments, we found that the addition of the acid-isopropanol solution to dissolve the blue crystals formed after MTT reduction

TABLE 2

RESPONSIVENESS OF DA1.2 AND 5/10.14 CLONES TO GROWTH FACTORS, LYMPHOKINES, MITOGENS, AND SERA

Factor	DA1.2	5/10.14
WEHI-3 CM	+ + + +	+ +
L-cell CM	−	+ + + +
Multi-CSF/IL-3	+ + + +	+ +
GM-CSF (murine)	+ + + +	+ + +
GM-CSF (human)[a]	−	−
CSF-1 (murine)	−	+ + + +
CSF-1 (human)	−	+ + + +
G-CSF (human)[a]	−	−
H1 (human)[a]	−	−
EPO (human)	+ +	−
EPO (murine)	+ +	−
IL-2 (human)	−	−
IFNγ (human)	−	−
Pluripoietin α (human)[a]	−	−
LBGF (human)[a]	−	−
S.F. (human)[b]	−	−
Concanavalin A	−	−
Phytohemagglutinin	−	−
Pokeweed mitogen	−	−
Bovine serum[c]	−	−
Rabbit serum[c]	−	+
Horse serum[c]	−	+
Human serum[c]	−	−
LPS	−	−
PMA	−	−

[a] 5637 conditioned medium (19–22).

[b] Synergistic factor (27).

[c] Over the serum concentration range 50 to 12.5%, the MTT assay results may not be evaluated due to formation of precipitates (as discussed in the text). Visual examination of the wells before reading MTT reduction indicated that the serum did not stimulate a proliferative response. At lower serum concentrations, precipitation did not interfer in the reading of the MTT reduction. N.T. = Not tested.

resulted in some precipitation of serum proteins. This problem was solved by reducing the amount of FBS present in the microtiter wells before addition of the acid-alcohol solution. Thus, the DA1.2 cells are tested using only 5% FBS, in a volume of only 50 μl, whereas 5/10.14 cells use only 1% FBS during the 5-h incubation step with MTT solution before reading O.D. These percentages of FBS do not affect the proliferative response. Adult sera precipitate more heavily in acid-isopropanol, and there is considerable variation between FBS lots. Therefore, when setting up these assay systems, one must empirically adjust the FBS concentration to avoid interferring precipitation during the reading of the assay.

The final concentration of MTT solution added to the cells should be about 10%. However, we have found that addition of MTT solution up to 30% does not affect the assay results.

We have found that changing pipette tips or rinsing the pipette tips with fresh medium between each doubling dilution step gives much better reproducibility. Also, the type of microtiter plate used may be an important variable in obtaining reproducible results. We found that microtiter plates that had a loose-fitting lid (Linbro no. 76-035-05) allowed more evaporation

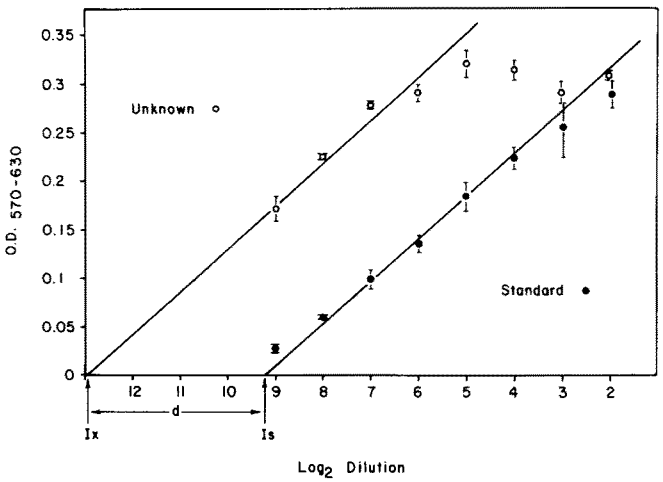

FIG. 3. Quantitation of CSF-1 Activity. Activity is always determined relative to a standard present in the same assay. Quantitation can be conveniently carried out on plots of response vs. $\log_2$ (dilution), displaying both standard and unknown, using the formula:

$$a_x = 2^d \times a_s$$

a_x and a_s are activities of unknown and standard, respectively; d is the horizontal distance between two parallel lines drawn through the steepest (central) portions of the sigmoidal dose-response curves, and are most easily measured between the lines' intercepts on the abscissa: $d = I_x - I_s$ with I_x and I_s being the intercepts for unknown and standard, respectively.

during the incubation step than plates that had a tighter-fitting lid (Linbro no. 76-003-05). Inasmuch as the final well volume was only 50 µl in our DA1.2 assay, the reproducibility of the assay was dramatically affected. In contrast, the larger volume (100 µl) of the 5/10.14 assay made it insensitive to the type of plate used.

Target cell lines used in any bioassay should be free of *Mycoplasma sp* (26). We routinely use two methods to detect *Mycoplasma sp:* (1) microbiological culture, and (2) an assay that utilizes mycoplasma-mediated cytotoxicity of a purine analog, 6-methylpurine deoxyriboside (6MPDR; ref 26). If *Mycoplasma sp* is found, infected cells can usually be treated and cured in 4 to 6 wks with BM-cycline (Boehringer Mannheim, West Germany) at doses kept just below cytotoxic levels, which are determined empirically for each cell type. The treated cells are then passaged drug-free several times before retesting for mycoplasma infection.

It is necessary to characterize the responsiveness of a cell line as fully as possible. By doing so, assay procedures can be developed that will identify specific hematopoietic growth factors in unknown samples. For example, a response with 5/10.14 cells, but not with DA1.2 cells, indicates only CSF-1 to be present in an unknown. In contrast, a strong response with DA1.2 cells and weak response with 5/10.14 cells, indicates Multi-CSF or GM-CSF and eliminates CSF-1. Having additional cell lines with fully characterized responses will allow the differential identification of Multi-CSF from GM-CSF and other hematopoietic growth factors.

As part of the characterization of DA1.2 cells for response to known HGF, lymphokines, and mitogens, it was found that both crude murine and purified (recombinant) human erythropoietin were active (EPO, Table 2). This interesting observation has been reported more fully in another paper (28).

Finally, it is imperative that each laboratory determine its own optimal conditions. We have given detailed instructions on the proper procedures to follow to establish and optimize hematopoietic growth factor assays. Our methods use a nonradioactive means to measure the proliferative response, which are similar to methods using more conventional [^{3}H]TdR assays. The colorimetric MTT assay is rapid and does not require expensive scintillation or gamma counters. However, use of an automated microtiter plate reader does add an additional cost to this assay. This cost is somewhat offset by the speed with which these machines read the samples. An entire 96-well plate can be read within 1.5 min. An additional advantage is that microtiter plate readers can be commercially interfaced with computers for analysis of data, making precise quantitation and readout possible.

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