Primary Bioassay of Human Myeloma Stem Cells

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ABSTRACT The ability to clone primary tumors in soft agar has proven useful in the study of the kinetics and biological properties of tumor stem cells. We report the development of an in vitro assay which permits formation of colonies of human monoclonal plasma cells in soft agar. Colony growth has been observed from bone marrow aspirates from 75% of the 70 patients with multiple myeloma or related monoclonal disorders studied. Growth was induced with either 0.02 ml of human type O erythrocytes or 0.25 ml of medium conditioned by the adherent spleen cells of mineral oil-primed BALB/c mice. 5–500 colonies appeared after 2–3 wk in culture yielding a plating efficiency of 0.001–0.1%. The number of myeloma colonies was proportional to the number of cells plated between concentrations of 10^5–10^6 and back-extrapolated through zero, suggesting that colonies were clones derived from single myeloma stem cells. Morphological, histochemical, and functional criteria showed the colonies to consist of immature plasmablasts and mature plasma cells. 60–80% of cells picked from colonies contained intracytoplasmic monoclonal immunoglobulin. Colony growth was most easily achieved from the bone marrow cells of untreated patients or those in relapse. Only 50% of bone marrow samples from patients in remission were successfully cultured. Tritiated thymidine suicide studies provided evidence that for most myeloma patients, a very high proportion of myeloma colony-forming cells was actively in transit through the cell cycle. Velocity sedimentation at 1 *g* showed myeloma stem cells sedimented in a broad band with a peak at 13 mm/h. Antibody to granulocyte colony-stimulating factor did not reduce the number or size of the colonies. Increased numbers of myeloma colonies were seen when the marrow was depleted of colony-stimulating factor elaborating adherent cells before plating. This bioassay should prove useful in studying the in vitro biological behavior of certain bone marrow-derived (B)-cell neoplasia. In addition, systematic and predictive studies of anticancer drug effects on myeloma stem cells should now be feasible.

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INTRODUCTION

Multiple myeloma has served as a valuable model neoplasm in both mouse and man (1). Studies of myeloma immunoglobulin (M-component) synthesis and metabolism have been applied to quantitate the total body number of myeloma cells and to follow changes in tumor mass with treatment. Such serial tumor kinetic studies, and those of the tritiated thymidine labeling index of the tumor, have provided important insights on the kinetics of growth and regression of myeloma and on approaches to treatment (1, 2). However, such studies do not directly assess the key compartment of the tumor, the tumor stem cells. Tumor stem cells provide the basic cell renewal system of the tumor, and are necessary to provide the colonizing property of a neoplasm capable of metastasis.

Usually, tumor stem cells have been assessed functionally in animals with various in vivo transplantation assays or with in vitro colony-forming assays. Such animal studies indicate that tumor stem cell assays can be used to study the properties of tumor stem cells and to delineate differences in individual sensitivities to chemotherapeutic agents (3–6). Furthermore, cloning systems (particularly those developed for culture of macrophage-granulocyte precursors) have proven ideal for the detection and characterization of regulatory factors controlling cell growth and differentiation (7–10).

Although primary explants of plasma cells in transplantable mouse myeloma have been successfully cloned in soft agar (4, 11, 12), the in vitro cultivation of human plasma cells has met with little success (13–16). Using methods similar to those he described

for in vitro cultivation of mouse myeloma cells, Park (15) was able to culture human myeloma cells in soft agar. However, he was unable to establish linearity between numbers of cells plated and the number of colonies formed, and thus could not use this technique as an assay system. The lack of an in vitro bioassay for myeloma stem cells has hampered the study of the natural history of myeloma and the quantitation of myeloma stem cell mass.

This paper descirbes a useful in vitro bioassay for human myeloma colony-forming units in culture (M-cfu-c)[1] which we have developed and applied successfully to the study of patients with multiple myeloma and related monoclonal bone marrow-derived (B)-cell neoplasms. Additionally, studies characterizing myeloma colony-forming cells and their growth requirements are reported.

METHODS

Patient studies. Patients with well-documented multiple myeloma, macroglobulinemia of Waldenström, or benign monoclonal gammopathy, and normal volunteers were selected for study. Detailed clinical and immunologic criteria for diagnosis and clinical staging of myeloma were as described previously (17). Studies which are carried out routinely in our laboratory for case classification in monoclonal neoplasia include immunoelectrophoresis and immunoquantitation of serum and urine M-components, radioimmunoassay measurement of the in vitro synthetic rate of M-component by bone marrow plasma cells (2, 18), and measurement of the tritiated thymidine ([^{3}H]Tdr) labeling index of marrow plasma cells by the high-speed scintillation autoradiography technique (19). Patients are studied during initial staging and before any treatment, as well as at various intervals after treatment. Total body tumor cell number was calculated from M-component measurements, and clinical staging was accomplished by the method of Durie and Salmon (17) and stored along with serial entries using a General Electric time-sharing network (General Electric Information Services, Main Headquarters, Rockville, Md.) which accurately quantitated myeloma cell mass changes with chemotherapy. Remission was defined as a 75% reduction in total body myeloma cell number. Relapse was defined as a 50% increase in myeloma cell mass over the remission level. Myeloma patients were treated every 3–4 wk with intermittent 4-day pulse courses of combinations of cycle-nonspecific drugs (e.g., melphalan, cyclophosphamide, or doxorubicin hydrochloride) plus prednisone with or without vincristine. Bone marrow samples for in vitro studies on patients receiving chemotherapy were obtained 3–4 wk after the previous course of chemotherapy.

Collection of cells. Bone marrow cells were obtained from patients or normal volunteers by sternal or iliac puncture after informed consent was obtained. Cells were aspirated into a heparinized syringe, mixed in an equal volume of 3% dextran-saline, and sedimented at room temperature for 45 min. The cells in the supernate were collected after centrifugation at 150 *g* for 10 min and washed twice in Hanks' balanced salt solution (HBSS) with 10% heat-inactivated fetal calf serum. The viable nucleated cell counts determined in a hemocytometer using trypan blue were routinely >95%. Bone marrow differential counts were performed on slides prepared with cytocentrifuge and stained with Wright-Giemsa stain.

Culture assay for M-cfu-c. Colonies derived from myeloma cells were grown in the presence of a 1-ml agar feeder layer in 35-mm Falcon petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Two types of feeder layers were prepared. The first was made by incorporating 0.02 ml of human type O+ washed erythrocytes in modified McCoy's 5a medium (Grand Island Biological Co., Grand Island, N. Y.) containing 0.5% agar (Bacto, Difco Laboratories, Detroit, Mich.) to a final volume of 1.0 ml. The modified preparation of McCoy's 5a medium consisted of 15% heat-inactivated fetal calf serum and a variety of nutrients as described by Pike and Robinson (20). Immediately before use, 10 ml of 3% tryptic soy broth (Grand Island Biological Co.), 0.6 ml asparagine (0.6 mg/ml), and 0.3 ml DEAE-dextran (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) were added to 40 ml of the enriched medium.

The second feeder layer utilized 0.25 ml of conditioned medium in 0.5% agar and enriched McCoy's 5a medium. The conditioned medium was prepared from the adherent spleen cells of BALB/c mice which had been primed with 0.2 ml of mineral oil injected intraperitoneally 4 wk previously. This was based on a protocol of Namba and Hanoka for in vitro cultivation of mouse myeloma cells (21). The adherent spleen cells were obtained as follows: the spleens were teased with needles to form a single cell suspension, and 5×10^6 cells were placed in 60-mm Falcon tissue culture dishes in RPMI 1640 medium (Grand Island Biological Co.) containing 20% horse serum. A 2-h incubation was used to permit cellular adherence. Subsequently, the dishes were rinsed three times in cold phosphate-buffered saline. Cells were then incubated for 3 days at 37°C in 5 ml of RPMI 1640 with 15% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (2 mg/ml), and glutamine (2 mM). The conditioned medium was decanted and centrifuged at 400 *g* for 20 min. The supernate was passed through a 0.45-μm Nalgene filter (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) and stored up to 1 mo at −20°C.

Bone marrow cells to be tested were suspended in 0.3% agar in CMRL 1066 medium (Grand Island Biological Co.) supplemented with 20% horse serum, penicillin (100 U/ml), streptomycin (2 mg/ml), glutamine (2 mM), $CaCl_2$ (4 mM), insulin (3 U/ml), asparagine (0.6 mg/ml), and DEAE-dextran (0.5 mg/ml) to yield a final concentration of 5×10^5 cells/ml. Freshly prepared 2-mercaptoethanol was added at a concentration of 50 μM immediately before plating the cells (22). 1 ml of the resultant mixture was pipetted onto the 1-ml feeder layer. Cultures were incubated at 37°C in 5% CO_2 in a humidified atmosphere with no additional feeding. Cultures were examined in an inverted phase microscope at ×100 and 200.

Final colony counts were made 14–21 days after plating. Inasmuch as cultures containing erythrocytes were quite opaque, erythrocyte lysis was completed by addition of 0.5 ml of 3% acetic acid as necessary before scoring.

Examination of cells in colonies. Individual colonies were removed from the dish using a fine capillary pipette and were suspended in a drop of heat-inactivated fetal calf serum. Colonies were air-dried 3–4 h. Cells were stained routinely with Wright-Giemsa, 0.5% orcein in 60% acetic acid, peroxidase (23), and methyl green pyronin (24).

[1] Abbreviations used in this paper: CSF, colony-stimulating factor; G-cfu-c, granulocyte colony-forming units in culture, [^{3}H] Tdr, tritiated thymidine; M-cfu-c, myeloma colony-forming units in culture.

For detection of cytoplasmic immunoglobulin, slides of individual colonies were air-dried and fixed in cold (−20°C) spectrophotometric grade acetone for 20 min. Slides were incubated for 45 min at 20°C with 0.025 ml of a 1:3 dilution of either fluorescein-conjugated rabbit anti-IgG, IgA, or IgM (Cappel Laboratories, Inc., Cochranville, Pa.). Cells were washed three times in phosphate-buffered saline at 20°C. Slides were examined in a fluorescent microscope with epi-illumination (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). 200 cells per slide were counted. Cells with moderate to strong fluorescence located only in the cytoplasm were scored as positive.

Determination of the percentage of cells in DNA synthesis by [^{3}H]Tdr killing. The [^{3}H]Tdr "suicide" method of Iscove et al. (25) was employed to measure the proportion of M-cfu-c in the S phase of the cell cycle. Briefly, samples of 2×10^6 cells, suspended in HBSS and 10% heat-inactivated fetal calf serum, were added to 1 ml of HBSS containing 40 μCi of [^{3}H]Tdr (23 Ci/mm, Amersham-Searle Corp., Arlington Heights, Ill.). Control samples were added to HBSS. Cell suspensions were then incubated for 30 min at 37°C, and washed twice with 20 ml of ice-cold HBSS containing 100 μg/ml of unlabeled thymidine and 10% fetal calf serum. Each suicide and control suspension was cultured in four replicate plates at a concentration of 5×10^5 nucleated cells per plate.

Cell separation by velocity sedimentation. Cell suspensions were separated by velocity sedimentation at unit gravity using the Staput method of Miller and Phillips (26). This technique separates cells primarily on the basis of size. Briefly, 1×10^8 cells, suspended in phosphate-buffered saline with 5% fetal calf serum were sedimented through a 15–30% fetal calf serum gradient in a cylindrical siliconized glass chamber 18 cm in diameter (Johns Scientific Co., Toronto, Ontario, Canada). Sedimentation was carried out at 4°C for 150 min, and the chamber was then drained. The first 250 ml, consisting of the fluid in the conical portion of the chamber, was discarded. The remainder of the gradient was collected in 50-ml fractions. Cells were centrifuged at 150 *g* for 10 min and washed. Nucleated cell counts for each fraction were made in a hemocytometer after dilution in 3% acetic acid, and cytocentrifuge preparations were made.

Preparation of nonadherent and nonphagocytic cell populations. Freshly washed bone marrow cells were further separated into adherent and nonadherent populations by the method of Messner et al. (27). Briefly, cells were allowed to adhere for 30 min to 60-mm plastic tissue culture dishes in RPMI 1640 medium and 15% fetal calf serum at 37°C. The supernate was decanted, and the adherence procedure was repeated three more times to provide the nonadherent cell population.

Bone marrow cells were depleted of phagocytic cells by placing 10^7 washed cells in tubes with 40 mg of carbonyl iron powder (Tridom-Fluka, Hauppage, N. Y.) and incubating the mixture in a shaking water bath at 37°C. After 30 min, the iron powder and iron-laden cells were attracted to the bottom of the flask with a magnet. The supernate was carefully poured off, and this step was repeated as often as necessary (generally two to three times) to remove all the iron powder and iron-laden phagocytic cells. The remaining cells in the supernate were considered nonphagocytic.

Assay for granulopoietic colony formation. The assay used for granulocyte colony formation by human bone marrow cells was described by Pike and Robinson (20).

RESULTS

Development and identification of colonies. Cell doublings were usually observed within 48 h of plating and clusters of 8–40 cells appeared within 5–10 days. Colonies (collections of more than 40 cells) appeared 14–21 days after plating. Cell lysis generally occurred 28 days after plating. During the 1st wk of incubation there was a progressive increase in the number of cells which commenced proliferation. Colonies consisted of 40 to several hundred large (>20 μm) round cells. Cells in myeloma colonies appeared to pile up on one another (Fig. 1A) as opposed to loosely aggregated cells in contaminating granulocyte colonies. Growth kinetics of human myeloma cells in agar were similar to those described by Park (15).

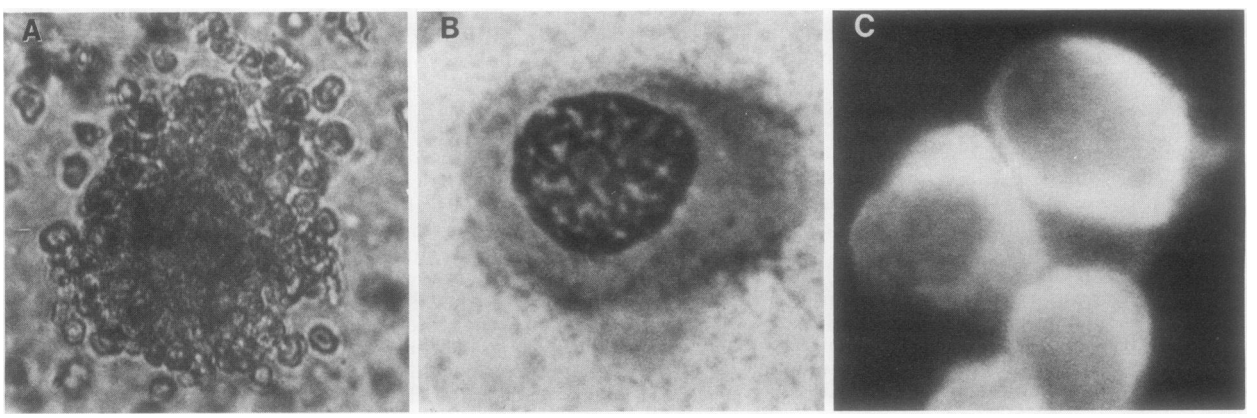

FIGURE 1 (A) A typical myeloma colony from a 21-day-old culture grown from the bone marrow of a patient in relapse (× 20). (B) A plasma cell picked from a 21-day-old culture and stained with Wright-Giemsa (× 100). (C) Cells from a colony grown from the bone marrow of a patient with IgG myeloma. Cells were fixed in acetone and stained directly with fluorescent anti-human IgG. Note the strong specific cytoplasmic fluorescence (× 100).

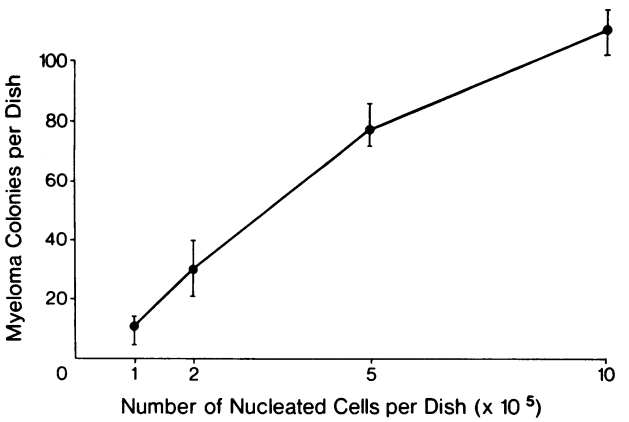

FIGURE 2 Linear relationship between colony formation and the number of nucleated bone marrow cells plated. Each point represents the mean of four dishes±SEM. These are the results of a typical experiment. Three other trials on different patients have yielded similar results.

The number of myeloma colonies ranged from 5 to 500 per plate, yielding a cloning efficiency of 0.001–0.1%. A linear relationship was obtained between the number of nucleated cells plated and the number of colonies found after 21 days (Fig. 2). The plot could be back-extrapolated through zero, suggesting colony origin from a single cell.

Cells from individual colonies when plucked from agar appeared to be plasma cells when examined by light microscopy after staining with Wright-Giemsa or methyl green pyronin (Fig. 1B). In Wright–Giemsa-stained preparations, the day 5–8 colonies were composed of a population of large mononuclear cells with bulky basophilic cytoplasm. However, colonies from older cultures contained mature plasma cells.

Cells were peroxidase-negative and incapable of phagocytosis of neutral red or latex particles. Immunofluorescent studies demonstrated that 60–80% of the myeloma cells of individual colonies had the cytoplasmic immunofluorescence specific for the monoclonal immunoglobulin present in the serum of the patient studied (Fig. 1C).

To study the cell renewal capability of the M-cfu-c, serial replating studies were carried out on myeloma colony plates from one patient (R. B.). In serial experiments, clusters and colonies of 2-, 5-, and 8-day incubations were plucked from the agar with a micropipette. The harvest of a given day was pooled, resuspended in medium, and passed through a 23-gauge needle for dispersion. The resulting single-cell suspension was then replated at 5×10^5 cells per plate over a fresh feeder layer. Despite the potential disruption and stem cell death which can result from such transfer experiments, clusters and colonies harvested as described above were capable of forming secondary myeloma colonies in the range of 100 colonies per plate, indicating some preservation of the primary colony-forming units in the culture system. In companion studies, washed cells plucked from myeloma colonies were incubated for varying times up to 24 h at 37°C in suspension culture along with tritiated leucine. The supernatant culture fluid from these cultures was then subjected to the solid-phase radioimmunoassay for intrinsically labeled IgG standardized in our laboratory (18). Cells from myeloma colonies were found to be capable of secreting IgG in quantities quite similar to myeloma cells from bone marrow samples (1, 2). The myeloma colonies were therefore operationally defined as arising from myeloma stem cells.

Factors affecting cell growth. Substitution of type A, B, or AB erythrocytes usually resulted in decreased numbers of colonies, although differences were not statistically significant. Mouse erythrocytes appeared to be cytotoxic. Lysates of O cells also supported growth. Depletion of residual colony-stimulating factor-producing leukocytes from erythrocytes by Ficoll-Hypaque (Pharmacia Fine Chemicals) or carbonyl iron methods did not affect colony growth (Table I).

Media conditioned with spleen cells of CD-1, DBA/2, and normal BALB/c mice were not so effective promoters of cell growth as conditioned media from oil-primed BALB/c mice. Conditioned media from WI 38 cells, MA 184 cells (bone marrow fibroblasts), or primary explants of human skin fibroblasts were unable to support cell growth. Bacterial lipopolysaccharide, mouse peritoneal exudate cells, mouse kidney fibroblasts, or frozen and thawed human leukemic bone marrow cells failed to stimulate myeloma cell growth. Serum from mice injected with endotoxin, bacterial antigens, or mineral oil did not allow colony growth. Autologous serum or urine from myeloma patients had no effect on colony growth.

The addition of ascorbic acid to culture of murine plasmacytoma cells has been reported by Park et al. to stimulate colony growth (4). Although vitamin C-rich CMRL enhanced cell growth, experiments using aged CMRL (depleted of vitamin C) failed to demonstrate the absolute requirement for vitamin C for myeloma colony growth (Table II).

Effect of clinical status on cell growth. Myeloma colony growth has been achieved in marrows from 83% of untreated patients or those in relapse (Table III). Only 50% of bone marrows from patients in remission on chemotherapy have formed myeloma colonies. Plasma cell colony growth was observed from the bone marrows of patients with benign monoclonal gammopathy or macroglobulinemia of Waldenström (Table III). The kinetics of plasma cell colony formation in these monoclonal disorders were

TABLE I
Effect of Substitution of Alternative Feeder Layers on Myeloma Colony Formation

	Number of colonies (per 5 × 10^5 cells)‡	
	Control (O cell)	Experimental
Erythrocyte feeder layers		
Substituted material (0.02 ml)		
O RBC* lysate	158±13.0	134±8.5
O RBC depleted of WBC with Hypaque-Ficoll gradient	40±8.0	49±5.8
O RBC depleted of WBC with Hypaque-Ficoll gradient followed by carbonyl-iron	40±8.0	41±4.6
A RBC	98±11.0	74±5.2
B RBC	98±11.0	80±2.5
AB RBC	98±11.0	88±6.1
BALB/c (mouse) RBC	98±11.0	0
	Control (oil-primed BALB/c)§	Experimental
Conditioned-medium feeder layers		
Cell source		
Normal BALB/c mice§	45±9.6	12±2.3
CD-1 mice (oil-primed)§	48±9.6	22±6.5
DBA/2 mice (oil-primed)§	45±9.6	8±2.3
WI38 (human fibroblasts)	45±9.6	2.0±2
MA 194 (human marrow fibroblasts)	45±9.6	1.8±1
Primary skin fibroblasts (human)	48±9.6	0

* RBC, erythrocytes; WBC, leukocytes.
‡ Mean±SEM.
§ Adherent spleen cells (Methods).

similar to those observed in myeloma. Normal bone marrow cells failed to grow plasma cell colonies when plated under these culture conditions. One of the normal donors had a 15% plasmacytosis 4 wk after an upper respiratory infection, but failed to form plasma cell colonies. The small number of colonies

TABLE II
Effect of L-Ascorbic Acid on Colony Formation of Human Myeloma Cells

Storage of medium used for plating	Myeloma colonies per 5 × 10^5 cells
1 mo at −20°C	91±4
1 mo at 4°C	58±5
1 mo at 4°C with 0.3 mM L-ascorbic acid	67±6

TABLE III
Relation of Patient Status to Monoclonal Plasma Cell Colony Growth

Patient status	Number of positive cultures/total studied subjects	Successful	Number (±SEM) of colonies/10^5 cells plated
		%	
Multiple myeloma			
Untreated	13/16*	81	105±15.0
Remission	12/24*	50	42±34.7
Relapse	21/25*	84	67±11
Macroglobulinemia of Waldenström			
Untreated	2/2	100	50±8
Benign monoclonal gammopathy	2/3	66	11±7.6
Reactive plasmacytosis	0/1	0	0
Normal	2/10‡	20‡	4±4*

* Comparison of no. of positive cultures/total subjects studied shows significantly higher rate of culture positivity in the combined group of untreated and relapse patients (34/41) vs. patients in remission (12/24) with the P value as determined by Fisher exact probability test <0.005.
‡ Granulocyte colony formation occurred in 2 of the 10 normal marrows tested, and are recorded as "positive." In all other subjects studied, colonies consisted of plasma cells.

that occasionally grew from normal marrow were peroxidase-positive and were granulocytes.

Proliferative state of M-cfu-c. High specific activity [^{3}H]Tdr was used to determine the percentage of M-cfu-c in the DNA synthetic (S phase) of the cell cycle (Table IV). [^{3}H]Tdr (40 μCi/ml) reduced colony survival to as little as 18% of control. Increasing the [^{3}H]Tdr concentration to 400 μCi/ml did not further increase the suicide fraction in either of two experiments. The addition of cold thymidine to [^{3}H]Tdr completely blocked the suicide effect in three experiments. The relationship of clinical status and various tumor kinetics parameters, including the suicide index, are summarized in Table IV. No suicide effect occurred in two patients (J. H. and V. W.) who had heavy infiltration of the marrow with myeloma cells and low [^{3}H]Tdr labeling indices.

Sedimentation velocity analysis of M-cfu-c. The sedimentation velocity of M-cfu-c was determined using the Staput apparatus. A representative experiment using bone marrow cells from an untreated myeloma patient is depicted in Fig. 3. Three additional experiments with cells from other myeloma patients have yielded similar results. In contrast to the nucleated cell profile obtained from normal bone marrow (28), myeloma bone marrow contained a large population of rapidly sedimenting cells. Myeloma stem cells sedimented as a single broad band with a peak sedimentation velocity of 13 mm/h. This value is in good agreement with that of Park who reported that M-cfu-c sedimented at 12 mm/h (15).

In an additional experiment, marrow cells from a second myeloma patient were separated on a Staput

TABLE IV

Relation of Clinical and Tumor Kinetic Parameters in Patients with Multiple Myeloma

Patient	M-Component	Clinical stage at diagnosis*	Status when studied	Bone marrow myeloma cells	Myeloma cell [^{3}H]Tdr labeling index	Total body myeloma cell number when studied‡	M-cfu-c 10^6 myeloma cells§	Plating efficiency	Fraction of M-cfu-c surviving [^{3}H]Tdr suicide
				%	%			%	
R. B.	IgGλ	IIA	Untreated	36	2	2.24×10^{12}	1,772±1.78	0.18	0.18
A. H.	IgGκ	IIIA	Untreated	36	14	1.81×10^{12}	180±30	0.02	0.33
L. Mc.	IgAκ	IIIA	Untreated	38	7	2.60×10^{12}	640±80	0.06	0.57
J. H.	κ-BJ	IIIB	Untreated	90	1	7.02×10^{12}	240±20	0.02	1.25
U. T.	λBJ (amyloid)	IB	Remission	4	1	0.98×10^{12}	190±23	0.02	0.25
E. F.	IgGλ	IIIA	Relapse	25	2	0.36×10^{12}	560±110	0.06	0.36
V. W.	IgAκ	IIIA	Relapse	73	1	3.69×10^{12}	300±50	0.03	1.21

* Clinical staging as described in reference 17.

‡ Calculated from initial cell mass from regression equation from reference 15, and corrected for change in M-component mass with remission or relapse.

§ All cultures were plated at 5×10^5 total nucleated marrow cells per plate, and are normalized to 10^6 with correction for plasma cell percentage. Plating efficiency for M-cfu-c was calculated in relation to the number of myeloma cells plated.

gradient, after which fractions were split, and cells were plated in either a granulocyte culture assay or the myeloma culture system. The number of granulocyte (G-) or M-cfu-c were evaluated in each fraction. The results are shown in Fig. 4. G-cfu-c sedimented much more slowly than M-cfu-c. There were approximately 10 times as many G-cfu-c in this marrow as M-cfu-c.

Effect of depletion of phagocytic or adherent cells. Although no exogenous source of colony-stimulating factor (CSF) is supplied in our culture system, adherent phagocytic cells of the bone marrow produce endogenous CSF that permits granulocyte growth (29). Therefore, bone marrow suspensions were depleted of either adherent or phagocytic cells, or both populations, before plating. In every case (Table V), depletion of the phagocytic or adherent cells increased the number of myeloma colonies seen per 5×10^5 cells. Thus, CSF-producing cells were not required for colony formation.

Effect of anti-CSF serum on myeloma colony formation. A rabbit antiserum to CSF prepared against mouse L cell CSF by Shadduck and Metcalf (30) was added to cultures to determine if myeloma

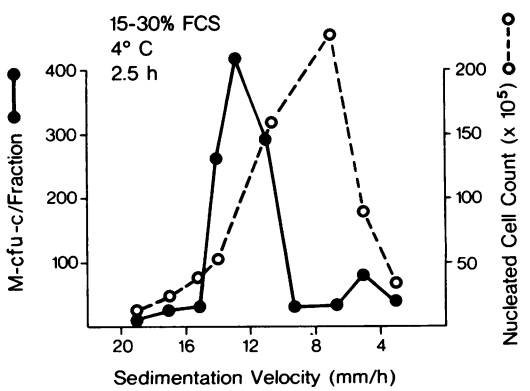

FIGURE 3 Results of a typical sedimentation velocity separation of cells from a bone marrow of a myeloma patient in relapse. 10^8 cells were sedimented through a 15–30% fetal calf serum (FCS) gradient at 4°C for 150 min. The dotted line represents the nucleated cell profile; the unbroken line the M-cfu-c profile.

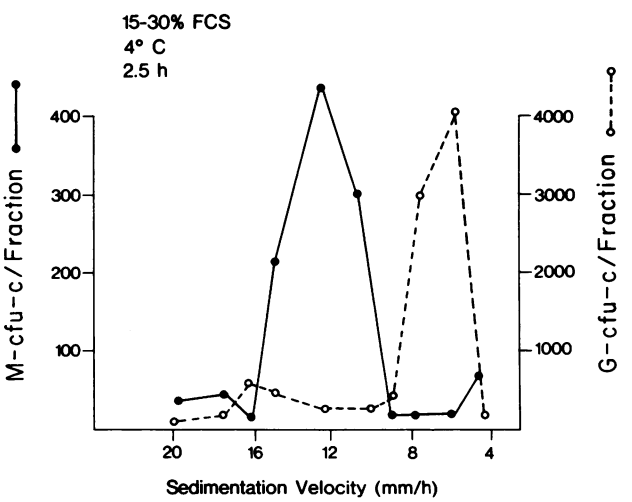

FIGURE 4 Results of a sedimentation velocity separation of cells from a bone marrow of a second myeloma patient in relapse. Cells were sedimented as described, fractions were split, and cells were grown in either a granulocyte or myeloma culture assay. The unbroken line represents the number of M-cfu-c per fraction; the dotted line represents the number of G-cfu-c per fraction. FCS, fetal calf serum.

TABLE V
Effect of Population Depletion on Myeloma Colony Formation

Population depleted	Exp	Number of M-cfu-c/5 × 10^5 cells plated*	
		Before depletion	After depletion
Adherent	1	11±7.2	92±8.3
	2	52±8.7	83±6.8
	3	65±8.6	101±11.2
	4	28±5.6	123±9.8
	5	44±4.8	220±28.5
Adherent phagocytic	5	48±8.1	80±22.0
Phagocytic	6	70±14.5	200±37.4

Mean±SEM of four plates.

colony formation was dependent on CSF and to eliminate the possibility that significant numbers of granulocyte colonies were growing in these cultures. The top portion of Table VI shows the results of a control experiment to determine the potency of the rabbit antiserum to CSF on granulocyte colony formation (30). Normal human bone marrow was plated in a standard Pike-Robinson granulocyte assay (20). The results indicated a 50% inhibition of colony formation at a serum dilution of 1:8. Normal rabbit serum did not depress granulocyte colony growth. The results of adding anti-CSF to bone marrow cells from myeloma patients in relapse cultured in our system are presented on the bottom of Table VI. Anti-CSF did not inhibit myeloma colony growth.

TABLE VI
Effect of Anti-CSF on Colony Formation by G- and M-cfu-c

Reciprocal of serum dilution	Number of colonies/5 × 10^4 cells	
	Anti-CSF	NRS*
G-cfu-c		
No serum	209±21.4	209±21.4
16	182±19.2	198±16.4
8	64±9.3	230±18.1
4	38±3.5	173±32.1
2	55±9.8	192±30.7
M-cfu-c		
No serum	366±25.7	366±25.7
16	331±29.8	322±28.8
8	345±13.1	320±28.1
4	380±30.5	365±60.0
2	316±40.8	292±15.9

Each point based on four plates±SEM. 0.1 ml of diluted antiserum was added to each plate.
* NRS indicates normal rabbit serum.

DISCUSSION

The present studies have shown that bone marrow cells from patients with multiple myeloma and related monoclonal disorders were capable of forming colonies of monoclonal plasma cells in soft agar. Growth was most easily achieved from bone marrows of untreated patients or those in relapse. A variety of morphological, histochemical, and immunofluorescent stains demonstrated that colonies consisted of plasma cells at different maturational stages. The immunochemical and cytochemical stains as well as the experiments with anti-CSF and depletion of bone marrow of CSF-producing cells indicated that the colonies did not consist of macrophages or granulocytes, nor did they require the stimuli for growth requisite for those myeloid cell types.

The fact that the number of colonies seen was directly proportional to the number of cells plated suggests that colonies were clones derived from single cells. This conclusion was supported by repeated observations of the development of colonies starting from a single cell. Plating efficiency, although low, was in the same range as that observed for granulocyte and erythroid colony-forming cells. Therefore, despite this low plating efficiency, our assay should prove useful in determining properties of myeloma stem cells and factors governing their proliferation in the same manner that in vitro assays have proven useful in elucidating G-cfu-c behavior. However, these low plating efficiencies could possibly lead to large sampling errors and ultimately affect estimates of the stem cell pool.

The nature of the potentiating effect of either erythrocytes or conditioned medium requires much further analysis. Metcalf (11, 12) has reported that intact mouse erythrocytes potentiated growth of murine plasmacytomas in vitro. However, this activity was lost when erythrocytes were lysed. In contrast, lysates of human erythrocytes supported myeloma cell growth. This might suggest that a different mechanism is involved in the potentiation of human plasma cell growth. Whether the factor(s) provided by erythrocytes and conditioned medium were similar remains to be determined.

In addition, the role of 2-mercaptoethanol remains unclear, although its helper effect in a variety of immunological reactions (31) and a clonal assay for mouse B lymphocytes has been well documented (22). It is possible that mercaptoethanol substitutes for a specific metabolite or induces the formation of a growth regulator in a fashion analogous to its promotion of release of CSF from lymphoid cells (32).

The absolute necessity of any of the additions to the CMRL overlay or the McCoy's medium feeder layer has not been documented. The use of horse

serum was prompted by reports of Park et al. (4) that high levels of fetal calf serum inhibited in vitro growth of murine plasmacytomas. We have noted a similar effect of fetal calf serum on human M-cfu-c growth. The use of human serum was avoided to minimize growth of granulocyte colonies (33).

We believe our in vitro technique will be useful in studying the growth kinetics of human myeloma. Prior studies from our laboratory have shown that the total body tumor mass in myeloma is in the range of 10^{12} cells at the time of diagnosis, with death occurring after two to three more doublings in total body tumor burden. Assuming that our in vitro colony assay does measure myeloma stem cells, then (based on our highest plating efficiencies of 0.2%) the minimum number of myeloma cells in the tumor stem cell compartment is in the range of 10^9 at the time of diagnosis. Our [^{3}H]Tdr suicide studies suggest that in most patients a very high proportion of M-cfu-c are in the cell cycle. If we assume that the total cell cycle duration is equal to twice the S-phase duration, then it would appear that the median fraction of myeloma stem cells in cycle in the patients studied was 72%. Of interest, there was no reduction in the number of M-cfu-c with [^{3}H]Tdr suicide in the two patients studied who had the highest concentrations of myeloma cells in the bone marrow (73 and 90%). The high concentration of total myeloma cells in these marrows may have transiently halted cycling or promoted entry into the G_0 compartment. The relatively high median [^{3}H]Tdr suicide index for M-cfu-c is in sharp contrast to the low proportion of marrow myeloma cells which appear to be in the S phase as measured by autoradiography with the [^{3}H]Tdr labeling index. The labeling index in untreated patients is generally <3% (34–36). By comparing the labeling index results to those of [^{3}H]Tdr suicide studies of M-cfu-c (Table IV) it appears likely that the M-cfu-c make up a sizeable proportion of those cells which can be identified as being in cycle with the labeling index.

The high suicide index and the relatively prompt appearance of 40-cell myeloma colonies in vitro suggest that human myeloma stem cells can proliferate rapidly. For example, if a myeloma colony contains 64 cells in 18 days, the in vitro doubling time is 3 days. Our previous marker kinetic studies based on M-component synthesis and metabolism indicated that myeloma grew in accord with Gompertzian kinetics. Despite the fact that the in vivo doubling time for a myeloma clone in the clinical phase of disease (5×10^{11}–5×10^{12} cells) was in the range of 2–6 mo, we could back-calculate from the growth or regression curves and predict a median initial tumor doubling time (one cell to two) of 1.88 days (2). Our in vitro colony-forming studies now provide independent evidence that when human myeloma stem cells are present in low cell concentrations, growth can proceed rapidly. It was of interest to observe that monoclonal colonies from macroglobulinemia and benign monoclonal gammopathy grew with equal vigor. Although we studied few cases, we speculate that differences in the in vivo environment or feedback regulation could account for the more indolent clinical behavior of those two neoplasms. Additional studies will be necessary to further assess plasma cell colony growth patterns in these latter disorders.

In addition to clarifying the cellular kinetics of myeloma, it appears that such assays will prove to have clinical importance. Specifically, in vitro tumor colony-forming assays have been used for detailed studies of drug and radiation sensitivity of mouse myeloma stem cells and have been predictive of in vivo results (6). In preliminary experiments, we have observed individual differences in sensitivity of human M-cfu-c from different patients to melphalan, 1,3-bis(2-chloroethyl)-1-nitrosourea, and doxorubicin hydrochloride. Previous studies from our laboratory relating total tumor mass staging to response to treatment and survival (17) suggested that tumor mass per se, while predictive of survival, was only of modest value in prediction of response to treatment. At that time we conjectured that the major measured predictive factor was drug sensitivity itself. Individualized studies of drug sensitivity of myeloma colonies may therefore have potential use for predictive cancer chemotherapy of multiple myeloma. We have now initiated a study to determine, both retrospectively and prospectively, if the in vitro survival of a patient's cells to drugs correlates with the clinical response to chemotherapy. A large number of such observations will be necessary to determine if the technique will be of value in predicting patients' response to treatment.

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REFERENCES

1. Salmon, S. E. 1973. Immunoglobulin synthesis and tumor kinetics of multiple myeloma. *Semin. Hematol.* **10:** 135–147.
2. Sullivan, P., and S. E. Salmon. 1972. Kinetics of tumor

growth and regression in IgG multiple myeloma. *J. Clin. Invest.* **51:** 1697–1706.
3. Ogawa, M., D. Bergsagel, and E. McCulloch. 1973. Chemotherapy of mouse myeloma: quantitative cell culture predictive of response *in vivo*. *Blood.* **41:** 7–15.
4. Park, C. H., D. Bergsagel, and E. McCulloch. 1971. Mouse myeloma tumor stem cells: a primary cell culture assay. *J. Natl. Cancer Inst.* **46:** 411–416.
5. Ogawa, M., D. Bergsagel, and E. McCulloch. 1971. Differential effect of melphalan on mouse myeloma (Adj. PC-5) and hemopoietic stem cells. *Cancer Res.* **31:** 2116–2119.
6. Ogawa, M., D. Bergsagel, and E. McCulloch. 1973. Sensitivity of human-murine hemopoietic precursor cells to chemotherapeutic agents assessed in cell culture. *Blood.* **42:** 851–858.
7. Bradley, T., and D. Metcalf. 1966. The growth of mouse bone marrow *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* **4:** 287–299.
8. Pluznik, D., and L. Sachs. 1966. The induction of clones of normal mast cells by a substance from conditioned media. *Exp. Cell Res.* **43:** 553–563.
9. Stephenson, J., A. Axelrod, D. McLeod, and M. Shreeve. 1972. Induction of colonies of hemoglobin synthesizing cells by erythropoietin *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **68:** 1542–1546.
10. Tepperman, A., J. Curtin, and E. McCulloch. 1974. Erythropoietic colonies in culture of human marrow. *Blood.* **44:** 659–669.
11. Metcalf, D. 1973. Colony formation in agar by mouse plasmacytoma cells: potentiation by hemopoietic cells and serum. *J. Cell Physiol.* **81:** 397–410.
12. Metcalf, D. 1974. The serum factor stimulating colony formation *in vitro* by murine plasmacytoma cells: response to antigens and mineral oil. *J. Immunol.* **113:** 235–243.
13. Johns, M., J. Fahey, and Z. Price. 1974. Long term establishment of a human plasmacyte cell line derived from a patient with IgD multiple myeloma. *J. Exp. Med.* **140:** 494–507.
14. Nilson, K., H. Bennich, G. Johanson, and J. Porter. 1970. Establishment of immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient. *Clin. Exp. Immunol.* **7:** 477–480.
15. Park, C. 1971. Studies of the growth characteristics of myeloma *in vitro*. Ph.D. dissertation, University of Toronto. 31–61.
16. Beckmann, H., R. Neth, H. Sohau, J. Ritter, K. Winkler, M. Garbrecht, K. Hausmann, and G. Rutter. Plasma cells and blast proliferation of leukemic human hemopoietic cells *in vitro*. *In* Progress in Differentiation Research. N. Muller-Berat, editor. American Elsevier Publishing Co., Inc., Elsevier North-Holland, Inc., New York, 383–395.
17. Durie, B., and S. Salmon. 1975. A clinical staging system for multiple myeloma. *Cancer.* **36:** 842–854.
18. Salmon, S., and B. Smith. 1970. Immunoglobulin synthesis and total body tumor cell number in IgG multiple myeloma. *J. Clin. Invest.* **49:** 1114–1119.
19. Durie, B., and S. Salmon, 1975. High speed scintillation autoradiography. *Science (Wash. D. C.).* **190:** 1093–1095.
20. Pike, B., and W. Robinson. 1970. Human bone marrow colony growth *in vitro*. *J. Cell Physiol.* **76:** 77–81.
21. Namba, Y., and M. Hanoka. 1972. Immunocytology of cultured IgM forming cells of mice. *J. Immunol.* **109:** 1193–1200.
22. Metcalf, D., G. Nossal, N. Warner, J. Miller, T. Mandell, J. Layton, and G. Gutman. 1975. Growth of B-lymphocyte colonies *in vitro*. *J. Exp. Med.* **142:** 1539–1545.
23. Todd, J. C., and A. H. Sandford. 1962. The Blood. *In* Clinical Diagnosis by Laboratory Methods. I. Davidson and D. Wells, editors. W. B. Saunders Company, Philadelphia. 131–132.
24. Weinberg, S., and F. Stohlman. 1976. Growth of mouse yolk sac cells cultured *in vivo*. *Br. J. Haematol.* **32:** 543–558.
25. Iscove, N., J. Till, and E. McCulloch. 1970. The proliferative state of mouse granulopoietic progenitor cells. *Proc. Soc. Exp. Biol. Med.* **134:** 33–37.
26. Miller, R., and R. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell Physiol.* **73:** 191–201.
27. Messner, H., J. Till, and E. McCulloch. 1973. Interacting cell populations affecting granulopoietic colony formation by normal and leukemic human marrow cells. *Blood.* **42:** 701–710.
28. Messner, H. J. T., II, and E. McCulloch. 1972. Density distribution of marrow cells from mouse and man. *Semin. Hematol.* **5:** 22–34.
29. Moore, M., W. Williams, and D. Metcalf. 1973. *In vitro* colony formation by normal and leukemic human hematopoietic cells. Interaction between colony forming and colony stimulating cells. *J. Natl. Cancer Inst.* **50:** 591–602.
30. Shadduck, R., and D. Metcalf. 1975. Preparation and neutralization characteristics of an anti-csa antibody. *J. Cell Physiol.* **86:** 247–252.
31. Heber-Katz, E., and R. Click. 1972. Immune response in vitro. V. Role of Mercaptoethanol in the mixed leukocyte reaction. *Cell. Immunol.* **5:** 410–418.
32. Parker, J., and D. Metcalf. 1974. Production of colony-stimulating factor in mitogen stimulated lymphocyte cultures. *J. Immunol.* **112:** 502–510.
33. Knudtzon, S. 1974. Growth stimulation of normal human bone marrow cells in agar culture by human serum. *Scand. J. Haematol.* **12:** 298–306.
34. Alberts, D., and D. Golde. 1975. DNA synthesis in multiple myeloma cells following cell cycle non-specific chemotherapy. *Cancer Res.* **34:** 2911–2914.
35. Salmon, S. E. 1975. Expansion of the growth fraction in multiple myeloma with alkylating agents. *Blood.* **45:** 119–129.
36. Drewinko, B., B. Brown, D. Humphrey, and R. Alexanian. 1974. Effect of chemotherapy on the labelling index of myeloma cells. *Cancer.* **34:** 526–531.