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PHYSIOLOGY OF CONTINUOUS BONE MARROW CULTURE DERIVED PERMANENT GRANULOCYTE-MACROPHAGE PROGENITOR CELLS

Joel S. Greenberger, M.D.

August 1983

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Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-82-C-2207

Dana-Farber Cancer Institute
Boston, MA 02115

SUMMARY

A careful analysis has been made of human T-cell lines for production of human Interleukin-3. The purpose of isolation of Interleukin-3 is to use it as a growth factor for unlimited self-renewal of hematopoietic progenitor cells which are capable of differentiating to mature neutrophillic granulocytes and granulocyte-macrophage progenitor cells. Several T-cell lines including K45, JURKAT, CEM, K230 have been screened for production of Interleukin-3 by assay of supernatant from cell lines for proliferation of mouse IL-3 dependent hematopoietic progenitor cell lines. Lines 45 and 230 produce low levels of activity. In contrast, IL-2 (T-cell growth factor) dependent human T-cell clones have been shown to produce high levels of a factor which is biologically similar to mouse IL-3. One human T-cell clone (B3) has been grown to quantities of 10^6-10^7 cells and fused with a 6-thyoguanine resistant variant of K45 to produce human T-cell hybridomas. In these and other experiments, supernatant from human T-cell hybridomas has been screened for production of human IL-3. Several positive clones have been found. Supernatants shown to contain biologically active human IL-3 activity is being screened for continued self-renewal of human hematopoietic progenitor cells derived from nonadherent cells removed from long-term bone marrow cultures. In attempts to improve the longevity of human long-term bone marrow cultures for generation of the target cells for these assays, modification of our original technique of hydrocortisone addition to intraoperative marrow has been developed. We have screened a variety of metabolic and monoclonal antibody treatments of human marrow prior to initiation of long-term marrow cultures in attempts to improve longevity. Isolation and characterization of several sources of potential human IL-3 is in progress. Biochemical analysis of the factor and methods for obtaining a stable cellular production source are being developed. Molecular biologic techniques for cloning the gene for human IL-3 will be applied in the next project period to attempt to isolate large quantities of the factor for studies with human cell lines *in vitro*. Analysis of the biologic properties of the cells *in vitro* will be carried out prior to studies testing their biologic effect in transfusion therapy of patients known to be granulocytopenic from total body irradiation.

FORWARD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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ANNUAL REPORT- DAMD-17-82-C-2207

Year 1 (8/1/32-8/1/83)

The initial goal of this proposal is to isolate stable cell lines producing human Interleukin-3, the growth factor required for continuous self-renewal in suspension cultures of human granulocyte-macrophage progenitor cell lines. Such cell lines upon isolation, successful freezedown and thawing, and demonstration that there is no evidence of detectable Epstein-Barr virus or other infectious viruses will then be tested for functions of bacterial phagocytosis and killing *in vitro* and ultimately for transfusion into patients recovering from total body irradiation or cytotoxic chemical exposure.

The primary goal (isolation of a cell line producing human Interleukin-3) has been approached by isolating clones of normal T-lymphocytes from human peripheral blood which are themselves dependent upon human Interleukin-2 for growth. These clones were isolated and screened for production of Interleukin-3 by several biologic assays. We have isolated several clones which are producing the factor (IL-3) and are preparing human hybridomas by fusing these T-cell clones with 6-thioguanine resistant (HGPRT-deficient) mutants of each of several human T-cell lymphoma cell lines. The T-cell lymphoma lines we have chosen are K45, derived from a human Sezary cell leukemia (Karpas, et al., *Leukemia Research* 1:35-49, 1977), a cell line called Jurkat, generously provided by Dr. Robert C. Gallo, National Cancer Institute (Defreitas, et al., *Proceedings of the National Academy of Sciences USA*, 79:6646-6650, 1982), and another T-cell line

called CEM, generously provided by Dr. Herbert Lazarus, Dana-Farber Cancer Institute. Each of these cell lines has been made resistant to 6-thioguanine or 8-azaguanine and thus is HGPRT deficient. Fusion is being carried out by ethylene-glycol selection of hybridomas in HAT selection medium and clones grown up for test for production of human Interleukin-3.

In addition, we are screening supernatants from a large number of T-cell hybridomas that have recently been produced by our colleagues in the field but have never been tested for the production of human Interleukin-3. These screenings are being carried out in an attempt to correlate possible other isolets of positive cell lines with those being derived in our laboratory. We have a strict understanding that a positive will result in transfer of that cell line to our laboratory for further study, amplification of the factor, and derivation of granulocyte-macrophage progenitor cell lines dependent upon the factor.

Screening for production of human Interleukin-3. Three assays have been carried out and have been shown to be positive with human T-cell clone B3.

1) Production of human GM-CFUc using nonadherent cells from human long-term bone marrow cultures. As shown in enclosed Table 1, nonadherent cells from human long-term bone marrow cultures plated at $2x10^5$ cells per plate generated 33, and 32 (greater-than-50-cell colonies) per plate when scored at day 14. In the absence of a known source of CSF, no colonies were detected (Table 1). For the positive control, the known source

of human CSF was monocyte-conditioned medium used as colony stimulating factor. To conclude that a cloned T-cell line produces human IL-3, one would need the following criteria. Supernatant from a cloned T-cell line that had been grown in the absence of any growth factor should be positive for production of GM-CFUc using nonadherent cells from long-term bone marrow cultures, also production of human BFUe in the presence of erythropoietin, and production of self-renewal of mouse IL-3 dependent multipotential hematopoietic stem cell lines recently shown by us to be clonal in origin and dependent on mouse IL-3. Finally, production of long-term self-renewal of nonadherent cells from human long-term bone marrow cultures should be observed.

As shown in Table 1, the first of these criteria have been fulfilled in that nonadherent cells of the T-cell clone B3 produced 95, and 90 greater than 50 cell colonies per $2x10^5$ nonadherent cells from long-term bone marrow cultures plated and scored on day 14. Several of the clones were positive but this one was known to be the most positive of the experiment. Control medium from the T-cell lines as shown in the bottom of Table 1 produced no colonies.

Experiments are continuing to produce stable hematopoietic cell hybrids between clone B3 and line K45 which is 6-thioguanine resistant. As shown in the acccompanying Table 2, the 6-thioguanine resistant resistant variant of K45 is stable and does not grow in HAT medium but does grow in the presence of 20ug/ml 8-azaguanine or 25ug/ml 6-thioguanine.

2) The second assay for determining whether cloned T-cell line B3 is producing Interleukin-3 or alternately a form of colony stimulating factor is to test for burst-promoting activity in the supernatant of clone B3.

These results will be forthcoming as the experiments are in progress. Several other clones have been shown to produce burst-promoting activity and these data will be ready for evaluation at the time of our next quarterly progress report.

3) The third and easiest assay for determining whether a clone is T-lymphocyte or human hybridoma producing Interleukin-3 is to test for stimulation of self-renewal of IL-3 dependent mouse hematopoietic progenitor cell lines.

Our laboratory is the only laboratory to have demonstrated permanent factor-dependent multipotential hematopoietic progenitor cell lines dependent for growth on mouse IL-3. As shown in the accompanying preprint, PNAS 1983 (in press), cell line B6SUta CL27 has been shown to require a minimum of 5 ng/ml mouse IL-3 for growth. Biologic characterization of several of the cell lines has been carried out, specifically with reference to cell phenotype and production of 20-alpha SDH in response to IL-3.

As shown in the accompanying recent reprint (Ihle, et al., Journal of Immunology, 129:1377-1383, 1982) we have demonstrated that cell line B6SUtA, although multipotential with the ability to produce (erythroid/ neutrophil/basophil-mast cell) lines from single cell derived colonies, is also positive for Thy1.1 surface antigen, and produces detectable quantities of 20-alpha SDH. The

cell line is absolutely dependent upon a source of IL-3 for proliferation in suspension culture and the cells die by 10-fold reduction every six hours after a 12 hour period following removal of IL-3 from the medium. As shown in the accompanying Figure 1, cell growth of this clone in various conditioned medias was shown to be absolutely dependent on IL-3. In the presence of L-cell CSF, human monocyte conditioned medium, or serum-free medium with or without fetal calf serium, there was no detectable production of cell growth in the absence of IL-3.

The assay for determining whether a human T-lymphocyte clone is producing human IL-3 is to culture 1.0×10^3 nonadherent cells of B6SUtA CL2/ in Linbro test wells in 1.0ml medium supplemented with 0.1% fetal calf serum and 0.1% of the unknown. The positive control is WEHI-3 cell-conditioned medium known to contain IL-3. The negative control is 0.1ml of serum-free medium. As shown in the accompanying Table 3, multiple supernatants from T-lymphocyte clones B3, B1, A3 have been tested and several are positive in this assay. Those which are positive include B3, shown to be positive in the human CSF assay (Table 1). Thus, there is correlation in two assays for determination of IL-3 activity and this provides further strong evidence that clone B3 is producing IL-3. As shown in the accompanying Table 3, supernatants from two sets of known T-cell hybridomas obtained are negative (My1-10, Fo 1-10). This indicates that the methods to establish T-cell hybridomas may not have been adequate for isolating IL-3 producers since clones of lymphocytes were not the source of the normal T-cell population, but rather pooled mitogen-stimulated T-cells. Our approach using a cloned B3 cell line is much more tedius

and the resultant hybrids will be longer in forthcoming, but it is anticipated that these hybrids will be stable and give us a better chance of having actual IL-3 cell production.

Also of great interest, cell line K230 is positive for IL-3 production. This is a permanent line recently obtained from our laboratory and is of great interest. Isolation of IL-3 is being carried out with this line. (Table 3)

A list of the manuscripts relating to this recent relating to this recent work is included.

II. Work on Developing Human Long-Term Bone Marrow Cultures.

A second goal of our contract proposal is to increase production of granulocyte-macrophage progenitor cells and multipotential hematopoietic stem cells from human continuous bone marrow cultures. To achieve these goals we have attempted to improve the long-term bone marrow culture system from the state at which it was developed in our laboratory and shown in the original grant proposal in our paper (Greenberg, et al., Blood, 58:724-731, 1981). To attempt to improve the culture system further, we have tested the effect of several additional agents on the longevity of human continuous marrow cultures.

As shown in the accompanying figures, there was no effect of Lithium Chloride (Figure 2), Sodium Selenite (Figure 3), Indomethacin (Figure 4) on improving hematopoiesis in long-term bone marrow cultures. In further experiments, we have tested for 1,25-OH-Vitamin D. This data looks promising, but it is preliminary. More recent experiments have tested for

separation of populations by monoclonal antibodies prior to establishment of long-term marrow cultures. As shown in Figures 5-6, monoclonal antibody treatment with B1 or J5 (CALLA) plus rabbit complement did not remove populations of cells that were inhibitory and thus produce longer continued hematopoiesis in the marrow cultures compared to the control cultures. Similar results were shown with monoclonal antibody B1, however B2 (Beta-2 microglobulin) and complement markedly decreased the production of hematopoietic cells due to the fact that this antigen marker is present on stromal and stem cells and the entire culture system was destroyed by this positive control antibody treatment. (Fig. 5-6)

As shown in Figures 7-8 cumulative production of cells was only decreased by B2 monoclonal antibody and complement treatment and not seen with B1, J5 or in more recent experiments with antibody to Ia or J2. The cumulative production of day seven GM-CFUc using B1, or J5 plus complement treatment, is shown in Figures 9 and 10. Only treatment with B2 (Beta-2 microglobulin) plus complement significantly reduced production of day 7 GM-CFUc. Similar data were shown for day 14 CFUc in which the cumulative production was only decreased by beta-2 microglobulin (B2) and complement production. (Fig. 11-12)

In further experiments, we have attempted to modulate the hydrocortisone concentration to further stimulate nonadherent cell production and found that 10^{-5} M hydrocortisone is optimal for the human marrow cultures. With each of several specimens, 4 from intraoperative hip preparations and 1 from a rib (Figure 13), we have seen an optimal production of adipocyte colonies in

the adherent stromal cells by 10^{-5}M hydrocortisone.

These data indicate that the optimal method for the human bone marrow culture system can be improved by using intraoperative marrow sources compared to aspirates, but that further improvements in the system and further reagents are forthcoming. We are currently testing several additional metabolic reagents to determine whether improvement in the system is possible.

A recent experiment involved the use of MY906, a monoclonal antibody derived at the Dana-Farber Cancer Institute and currently being tested as specific for the identification of CFU-GEMM, GM-CFUc monocyte-macrophage progenitor cells.

Treating human marrow single cell suspensions with this MY906 monoclonal antibody plus complement, it was possible to determine whether stromal cells were also removed by treatment with this monoclonal antibody, similar to the positive we obtained with B2. In this experiment MY906-positive cells were rosetted separated by Ficoll Hypaque density centrifugation after rosette with sheep red cells coated with the antibody. The MY906 negative population was thus lighter. We established long-term bone marrow cultures from these two populations as well as from undisturbed marrow cultures.

Although the result is preliminary at this point, 6 weeks ago when the experiment was established it was clear that the adherent cell population comprising the hematopoietic stem cells and "cobblestone" areas known to contain mouse CFUs (and presumed pluripotential stem cells for human long-term marrow cultures)

were greatly concentrated in the MY906-negative population. At this point, cumulative cell production in both populations has exceeded 10^7 cells/flask (increased significantly) but there is a greater proportion of hematopoietic progenitor cells produced by the MY906 negative cell cultures. These data indicate it may be possible to separate specific populations of human progenitor cells for enrichment of long-term bone marrow cultures to improve longevity and also to isolate progenitors that are likely to become permanent lines in human IL-3.

Goals for the second year of the contract.

Isolation of the appropriate T-cell hybridoma or T-cell line producing high quantities of IL-3 should be completed by this year. At that point a further investigation of the biologic properties of human long-term GM-CFUc lines dependent for growth upon this factor will be carried out. As outlined in the original proposal, during the second year we plan to do studies on phagocytosis and killing of microorganisms by the granulocyte-progenitor cell lines after they are induced to differentiate to polymorphonuclear leukocytes in vitro. We anticipate that cloning of the genes for the IL-3 from our T-hybridomas or t-cell lines should be possible within the third year, for amplification of large amounts in the range of mgs of protein. This should then enable us to produce large numbers of GM-progenitor cells for our first studies in vivo: First, transplanting the cells to diffusion chambers in irradiated mice or rats to characterize their biologic activity in vivo, and then to begin to explore the possibility of a human clinical trial, transfusing cells into patients who require bone

marrow stem cells.

Prior to any clinical trials, we will be testing for the presence of Epstein- Barr viral DNA, cytomegalovirus, and activation of human retrovirus genes, specifically those analagous to the HTLV recently described and isolated by Dr. Robert Gallo at the NCI.

TABLE 1: IN VITRO CFUc ASSAY

SUPERNATANT FROM T-CELL CLONES PLUS LTBMC CELLS	DAY 4		DAY 7		DAY 14	
	>50 CELLS	10-50 CELLS	>50 CELLS	10-50 CELLS	>50 CELLS	10-50 CELLS
$1x10^5$ cells						
Pos. control	0	6±1.4	0	5±1.4	1.5±2.1	1.5±2.1
Neg. control	0	0	0	0	0	0
B-1	0	58±22.6	3±1.4	121±24.7	33±1.4	25±10
B-3	0	62±12.7	1.5±.7	100±2	38±5.6	18±1.4
B-8	0	8±2.1	0	38±9	8±1.4	17±5
B-9	0	0	0	11±1.4	1.5±2.1	1.5±.7
C-3	0	2.5±2.1	0	14±5.6	1±1.4	1.5±2.1
C-8	0	48±12	0	19±5	12±2.1	7±3.5
E-10	0	0	0	50±25	10±6.3	13±.7
F8	0	0	0	0	0	0
Medium alone	0	0	0	0	0	0
$2x10^5$ cells						
Pos. control	0	14±2.1	5±1.4	64±8.4	33±7	15±3.5
Neg. control	0	0	0	0	0	0
B-1	0	0.5±.7	0	29±7	21±13	27±7.7
B-3	0	78±17	6±5	277±19	93±3.5	37±8
B-8	0	22±17.6	0	202±54	92±8.4	37±12
B-9	0	0	0	0	0	0
C-3	0	10±4	0	49±38	39±30	17±5
C-8	0	1±1.4	0	28±27	28±2	22±13
C-10	0	16±8	0	121±1.4	100±6	29±0
E-10	0	1±0	0	35±28	42±41	22±.7
F-8	0	32±0	4±1.4	339±59	88±8	20±.7
Medium alone	0	0	0	0	0	0

TABLE 2: HAT Medium Sensitivity and 8-Azaguanine Resistance of Cell Line K45

Line	Cell Number	Medium alone	Cell Number Growth at 7 Days	Medium with 8-Aza	
K45	10^5	RPMI+10% FCS	$>10^6$	RPMI+10% FCS +20ug/ml 8-Aza	N.G.
	10^4	"	$>10^6$		N.G.
	10^3	"	$\sim10^5$		N.G.
	10^2	"	$\sim10^4$		N.G.
	10	"	$\sim10^3$		N.G.
	10^5	HAT	$>10^6$		
	10^4	"	$>10^6$		
	10^3	"	$\sim10^5$		
	10^2	"	$\sim10^4$		
	10	"	$\sim10^2$		
K45 (8-Aza-R)	10^5	RPMI+10% FCS	$>10^6$	RPMI+10% FCS +20ug/ml 8-Aza	$>10^6$
	10^4	"	$>10^6$		$\sim10^6$
	10^3	"	$\sim10^5$		$\sim10^5$
	10^2	"	$\sim10^3$		$\sim10^3$
	10	"	$\sim10^2$		$\sim10^2$
K45 (8-Aza-R)	10^5	HAT	$<10^1$		
	10^4	"	N.G.		
	10^3	"	N.G.		
	10^2	"	N.G.		
	10	"	N.G.		

N.G. = no growth

TABLE 3: GROWTH OF B6SUtA (IL-3 DEPENDENT CELL LINE IN TEST SOURCES OF HUMAN IL-3 (STARTING 10^3 CELLS/WELL)

SOURCE OF CONDITIONED MEDIA: (PERMANENT CELL LINE)	GROWTH OF B6SUtA CELLS AT 14 DAYS (CELL NUMBER PER WELL)
Murine	
WEHI-3	$>10^5$
L929	0
None	0
Human	
K45	$\approx 10^2$
Jurkat	0
K230	$>10^{4*}$
(Human T-cell Clones, IL-2 Dependant)	
B3	$>10^{5*}$
B1	$>10^{5*}$
A3	0
(Isolated T-T Hybridomas)	
1 Isolate-1 (My)	0
2	0
3	0
4	0
5	0
6	0
7	0
8	0
9	0
10	0
1 Isolate-2 (Fo)	0
2	0
3	0
4	0
5	0
6	0
7	0
8	0
9	0
10	0

*Potential Source of Human IL-3

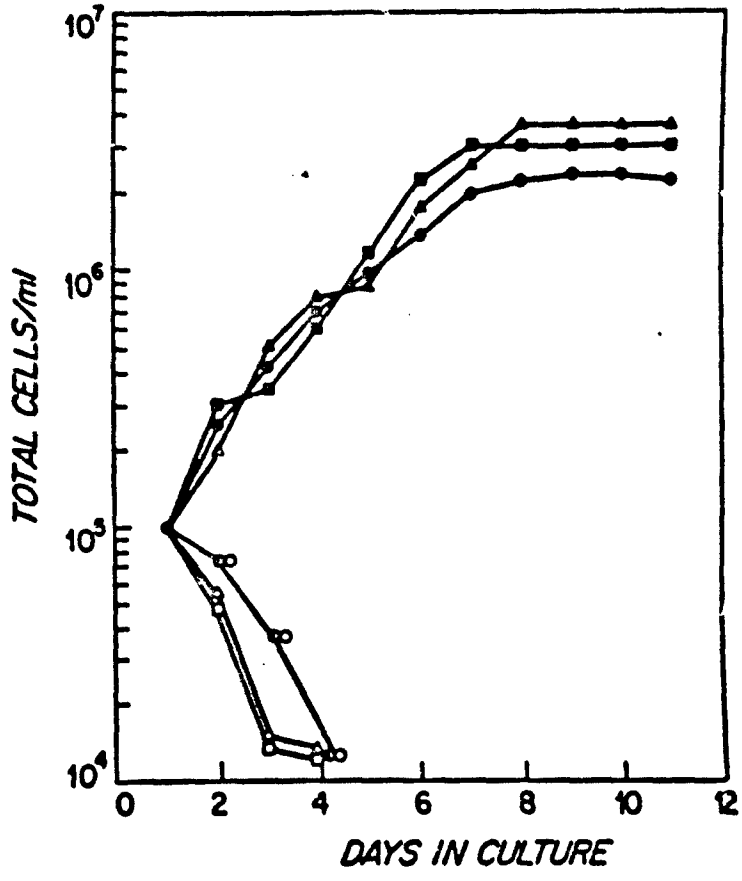
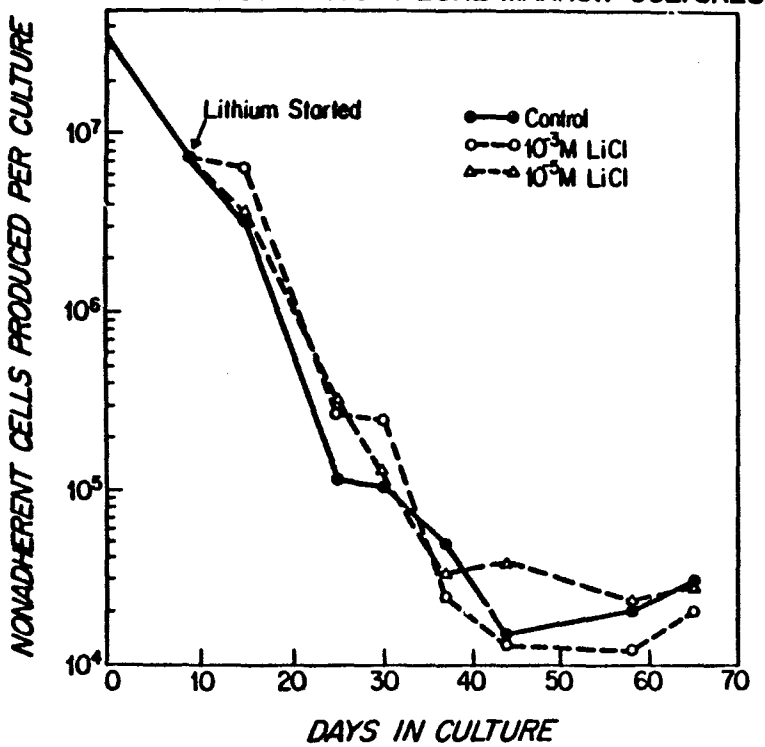


Figure 1: Growth of factor- dependent cell line B6SUtA cl 5 in medium supplemented with various conditioned medias or purified growth factors. Aliquots of 10^5 cells were suspended in 4.0 ml volumes of McCoy's 5A supplemented medium (23) with 10% fetal calf serum and: no additives (○); or addition of10% L929 cell coditioned medium as a source of CSF (□); 10% of a fraction of pokeweed mitogen spleen conditioned medium known to contain 10ng/ml IL-2, (△); 10% medium from 7 day old dense 8 week old long term bone marrow cultures grown in 20% FCS (22) (●); 10% WEHI-3 cell conditioned medium (●); 10% pokeweed mitogen spleen conditioned medium (▲) (18); or 10 ng/ml IL-3 purified according to published methods (■) (26). Results are presented as the mean for at least 3 plates at each point. The standard error of the mean was $\leq$15% of

EFFECT OF LITHIUM CHLORIDE ON HEMATOPOIESIS
IN HUMAN CONTINUOUS BONE MARROW CULTURES



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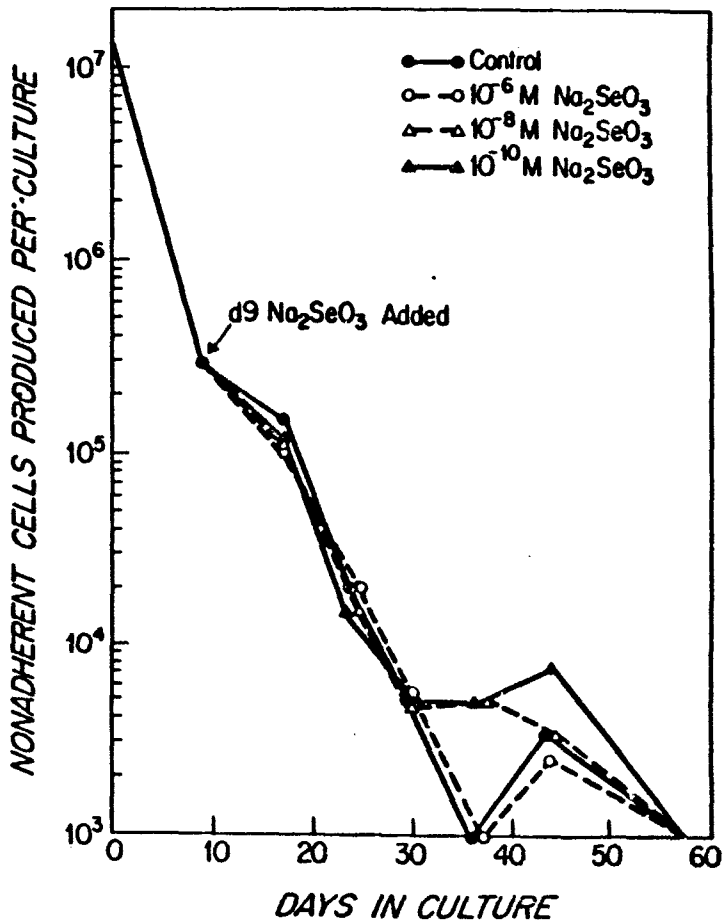
EFFECT OF SODIUM SELENITE ON HEMATOPOIESIS
IN HUMAN CONTINUOUS BONE MARROW CULTURES



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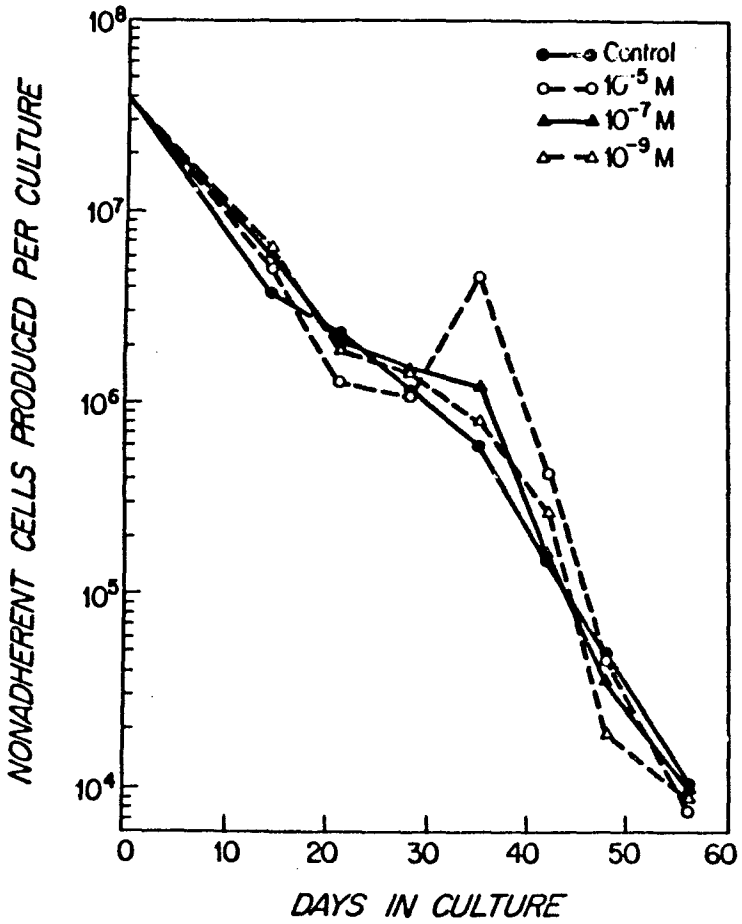
EFFECT OF INDOMETHACIN ON HEMATOPOIESIS
IN HUMAN CONTINUOUS BONE MARROW CULTURES



F4

EFFECT OF MONOCLONAL ANTIBODY AND
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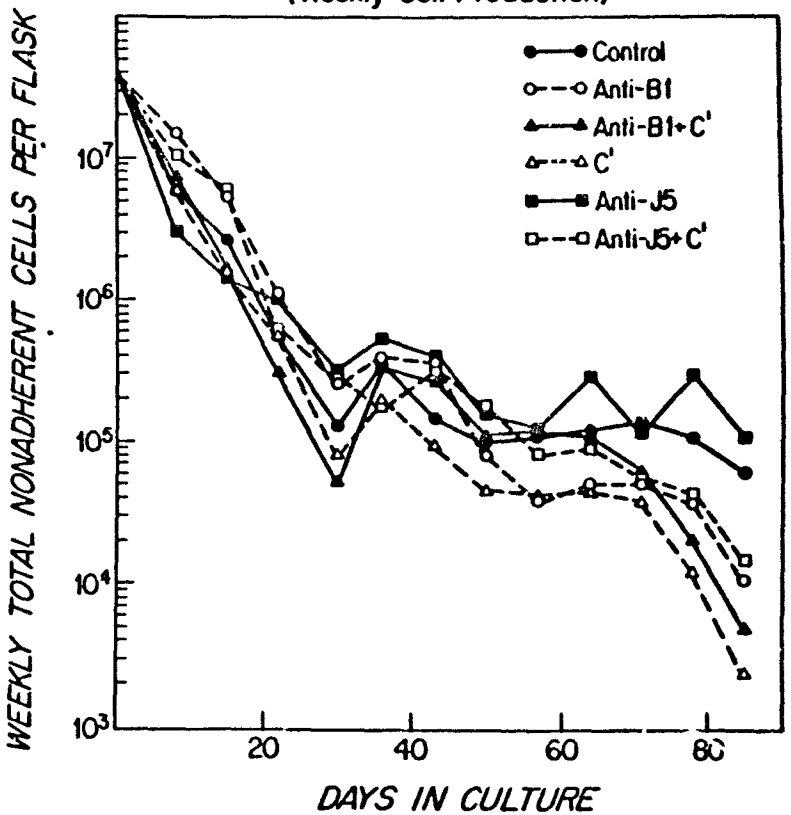
BONE MARROW CULTURES
(Weekly Cell Production)



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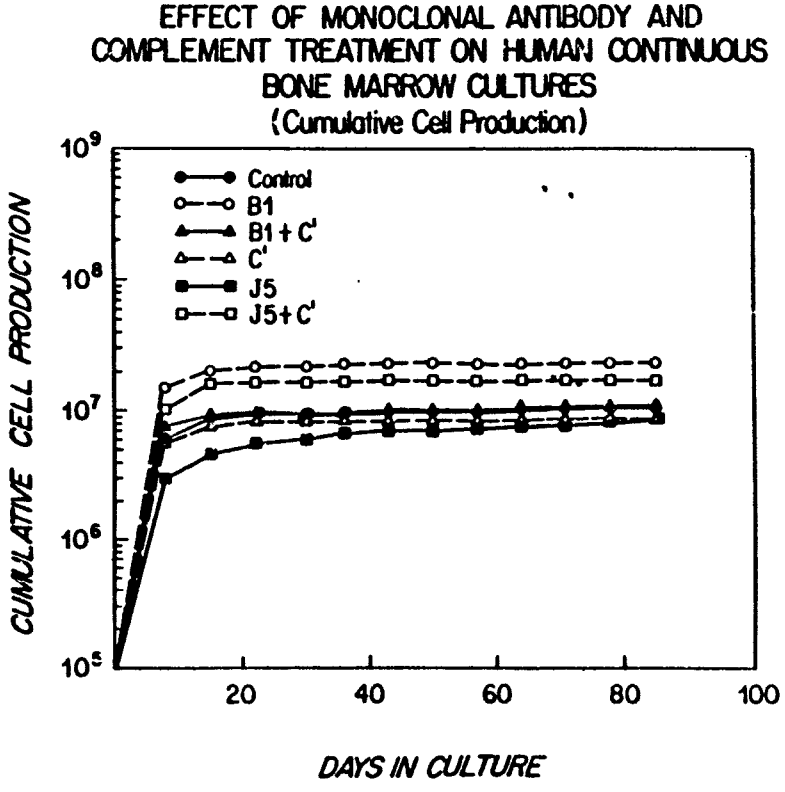
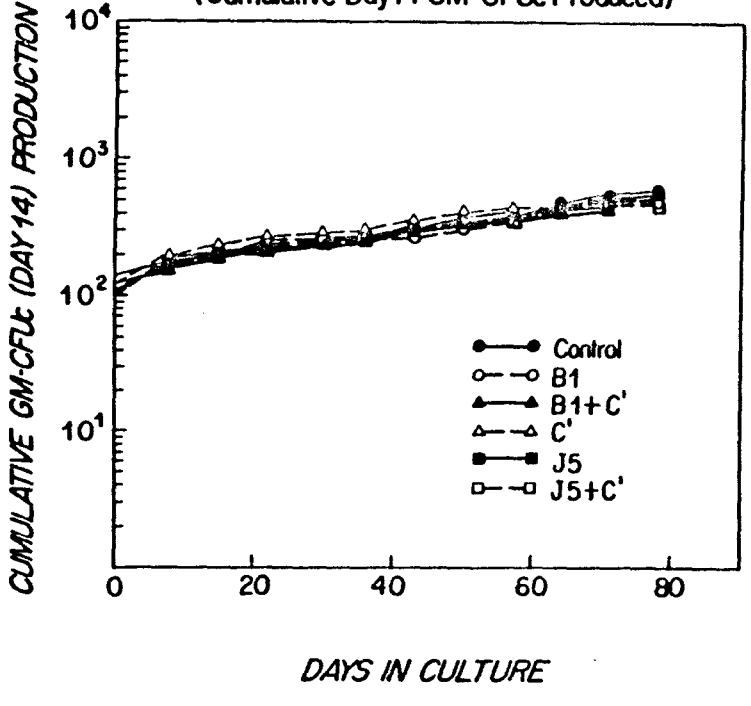

EFFECT OF MONOCLONAL ANTIBODY AND
COMPLEMENT TREATMENT ON HUMAN CONTINUOUS
BONE MARROW CULTURES
(Cumulative Cell Production)
CUMULATIVE CELL PRODUCTION
10^9
10^8
10^7
10^6
10^5
Control
B1
B1 + C'
C'
J5
J5+C'
20
40
60
80
100
DAYS IN CULTURE

F6

EFFECT OF MONOCLONAL ANTIBODY AND COMPLEMENT TREATMENT ON HUMAN CONTINUOUS BONE MARROW CULTURES

(Cumulative Day14 GM-CFUc Produced)



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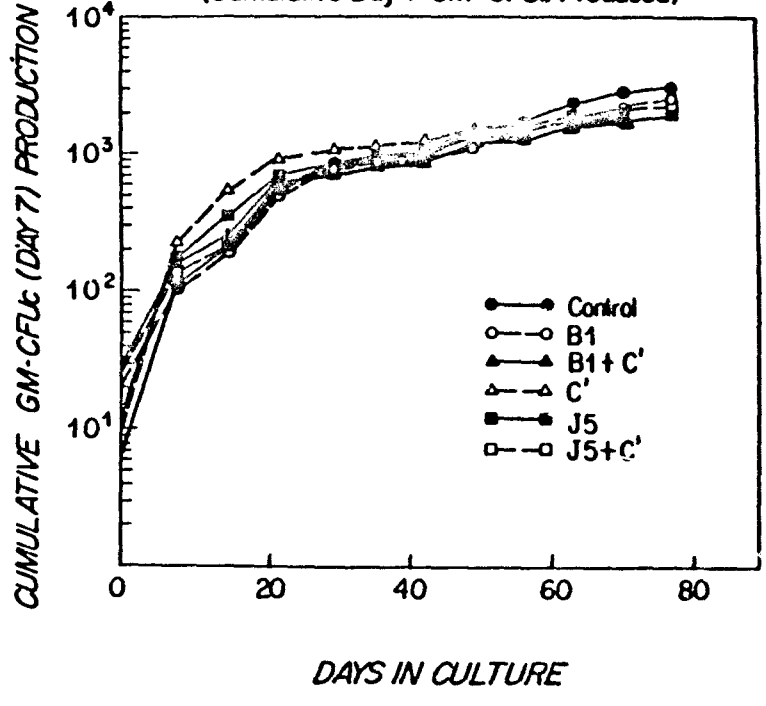
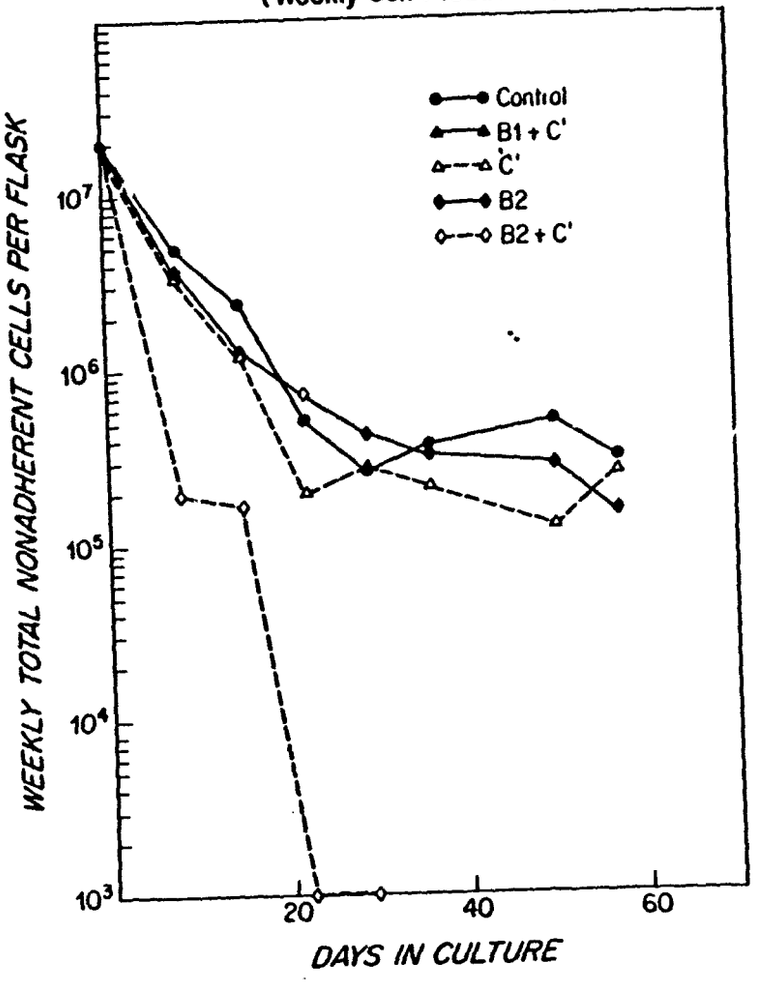
EFFECT OF MONOCLONAL ANTIBODY AND COMPLEMENT TREATMENT ON HUMAN CONTINUOUS BONE MARROW CULTURES

(Cumulative Day 7 GM-CFUc Produced)

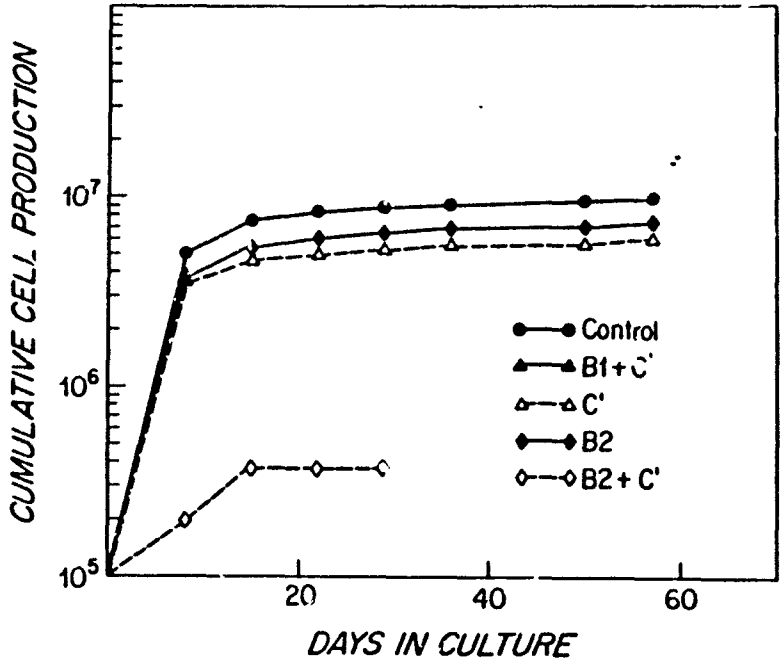


F8

EFFECT OF MONOCLONAL ANTIBODY AND COMPLEMENT TREATMENT ON HUMAN CONTINUOUS BONE MARROW CULTURES
(Weekly Cell Production)



EFFECT OF MONOCLONAL ANTIBODY AND COMPLEMENT TREATMENT ON HUMAN CONTINUOUS BONE MARROW CULTURES
(Cumulative Cell Production)



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EFFECT OF MONOCLONAL ANTIBODY AND COMPLEMENT TREATMENT ON HUMAN CONTINUOUS BONE MARROW CULTURES
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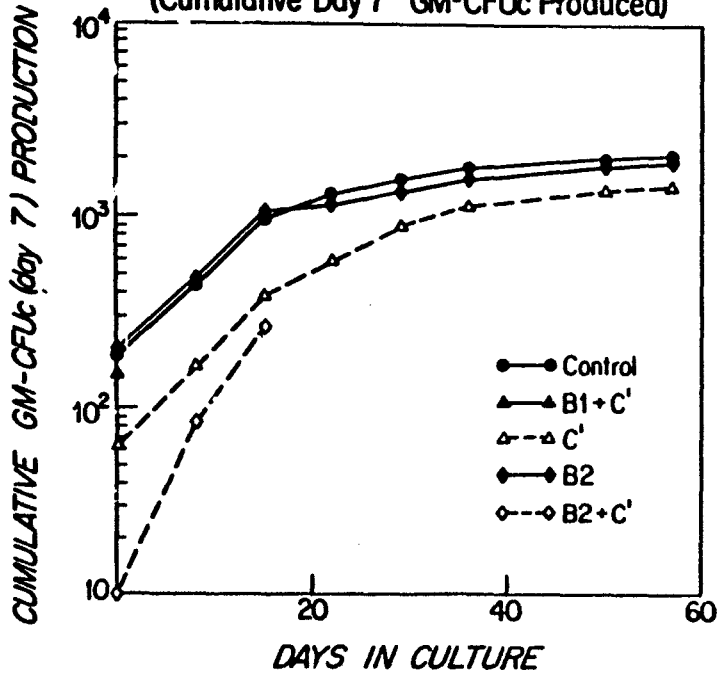
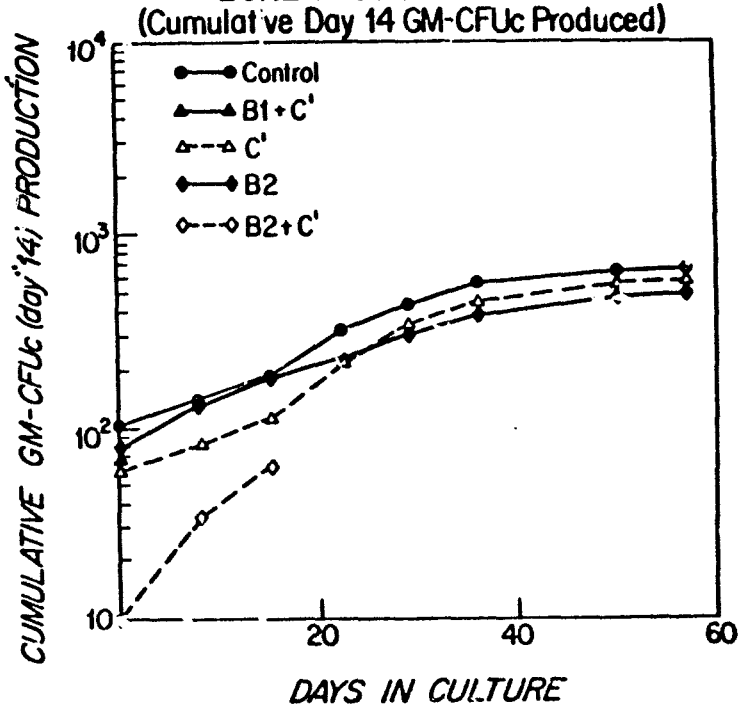


F11

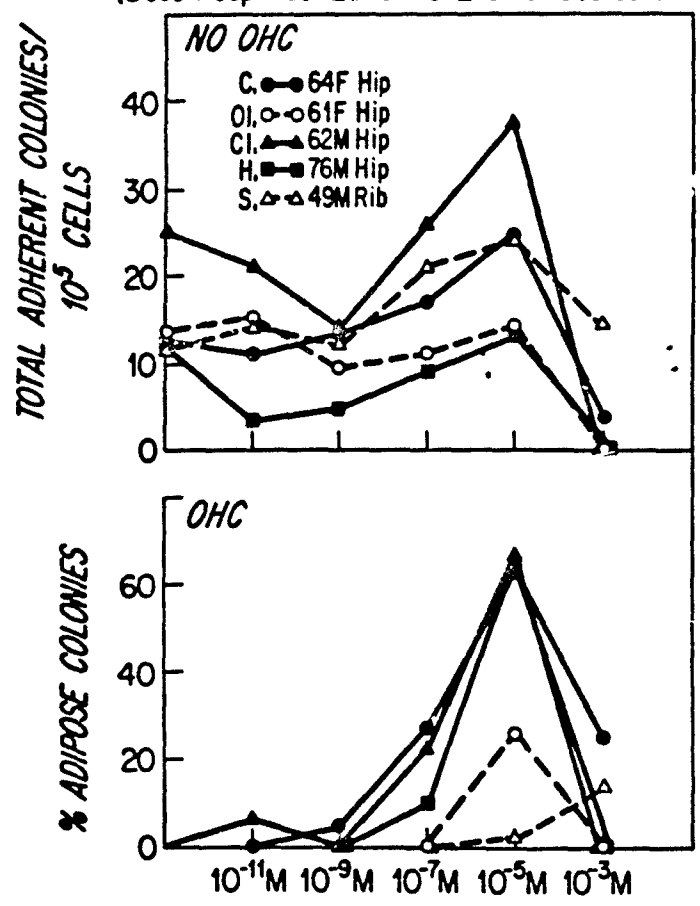
EFFECT OF MONOCLONAL ANTIBODY AND COMPLEMENT TREATMENT ON HUMAN CONTINUOUS BONE MARROW CULTURES
(Cumulative Day 14 GM-CFUc Produced)



F12

EFFECT OF HYDROCORTISONE CONCENTRATION ON MARROW ADIPOCYTE AND ADHERENT CELL COLONY FORMATION

(Dose Response 25% HS ± OHC Scored at d14)



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APPENDIX ABSTRACTS

1A. Upchurch, KS, Cohen, G, Greenberger, JS, Krane, SM. Hormonal responsiveness of continuous bone marrow-derived adherent cells: evidence for an osteoclast precursor. Blood, 60: Suppl. 1, p. 105a, 1982.

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Long-term cultures of murine bone marrow have been demonstrated by us to generate nonadherent cells that form permanent cloned factor-dependent granulocyte-macrophage progenitor cell lines with the capability for in vitro differentiation on purified stromal cells to polymorphonuclear leukocytes. Granulocytes derived from these lines demonstrate leukocyte functions in vitro including: phagocytosis, respiratory burst, superoxide generation, degranulation and bactericidal capacity. When injected at 10^7 cells per day over 5 days these cells protect total body irradiated mice from lethal bacterial infection. The growth-factor

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20. Abstract (continued)

required for self-renewal of mouse granulocyte progenitor cell lines is a 41,000 MW protein(s) produced by $Lyl^{+}2^{-}$ cloned inducer T-lymphocytes or the WEHI-3 cell line. Continuous human bone marrow cultures have been demonstrated by us to generate granulocyte-macrophage progenitor cells for 20 weeks and nonadherent cells establish lines of granulocyte-macrophage progenitor cells in medium conditioned by either of two available human T-lymphocyte-derived cell lines. We now propose to develop the system with human marrow cultures to a level suitable for clinical granulocyte-macrophage progenitor cell and granulocyte transfusion. Functional characteristics of uninduced and differentiation-induced human granulocyte-macrophage progenitor cells will be evaluated to measure respiratory burst activity, degranulation and bacterocidal capacity in vitro for a variety of known human opportunistic pathogens. Cryopreservation of granulocyte-macrophage progenitor cell lines, differentiated progeny and functional capacity of thawed cells will be tested. Cells will be tested for Epstein-Barr virus and graft versus host reactions using HLA incompatible human continuous bone marrow cultures. Methods will involve suspension culture of factor-dependent cell lines, continuous human bone marrow cultures, assays for granulocyte function, cryopreservation, assays of bacterocidal capacity in vitro, and assays for tumorogenecity of cell lines in nude mice and cell differentiation in diffusion chambers in irradiated mice.