## AD NUMBER

ADB233503

## NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Information; Oct 97. Other requests shall be referred to US Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702-5012.

## AUTHORITY

USAMRMC ltr dtd 4 Jan 2000

AD_____

GRANT NUMBER DAMD17-94-J-4465

TITLE: Megakaryocytopoiesis in Stem Cell Transplantation

PRINCIPAL INVESTIGATOR: Isaac Cohen, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

REPORT DATE: October 1997



TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | October 1997 | Annual (16 Sep 96 - 15 Sep 97) |

**4. TITLE AND SUBTITLE**

Megakaryocytopoiesis in Stem Cell Transplantation

**5. FUNDING NUMBERS**

DAMD17-94-J-4465

**6. AUTHOR(S)**

Isaac Cohen, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Northwestern University
Evanston, Illinois 60208-1110

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Distribution authorized to U.S. Government agencies only (proprietary information, Oct 97). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

The overall objective of this research proposal is to investigate several laboratory protocols to improve megakaryocyte production for future clinical use of an autologous megakaryocyte-rich product. Conditioned medium from bone marrow stromal cultures enhances the thrombopoietin-stimulated megakaryocyte production by at least 3-fold by a still unknown mechanism. We were able to rule out any role of stromal concentrations of thrombopoietin, interleukins 3 and 6, stem cell factor and heparan sulfates on this enhancing effect. In view of the synergistic activity of thrombopoietin and IL-3 that we previously reported, we investigated the effect of promegapoietin (Searle, R& D), a novel chimeric protein that is a multifunctional agonist of c-mpl and IL-3 receptors, on hematopoietic progenitors from bone marrow, umbilical cord blood and peripheral blood. Promegapoietin induced in bone marrow and peripheral blood similar synergistic increase in megakaryocyte production as thrombopoietin+IL-3 in a serum-containing as well as in a serum-free medium. Whereas umbilical cord blood was the most productive hematopoietic source for megakaryocytes, due to the high concentration of primitive megakaryocyte progenitors, the megakaryocyte productivity of peripheral blood is amply sufficient for clinical use of an *ex vivo*-expanded product. In view of the present unavailability of IL-3 for clinical use, promegapoietin appears to be the cytokine of choice to be considered for optimal megakaryocyte production in *ex vivo* expansion protocols.

**14. SUBJECT TERMS**

Breast Cancer

**15. NUMBER OF PAGES**

24

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Limited |

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

IC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

IC In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

IC For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jane Glen 9/24/97

PI - Signature Date

## Table of Contents



1 publication (reflecting work finished in year 02)

## INTRODUCTION

Following high-dose chemotherapy for the treatment of malignant disease, reinfusion of peripheral blood stem cells collected during treatment with growth factors usually results in delayed platelet engraftment as compared to neutrophil recovery (1-4), requiring patients to be submitted to repeated platelet transfusions. The overall objective of this research proposal is to investigate several laboratory protocols to improve megakaryocyte production for future clinical use of an autologous megakaryocyte-rich product. This material will supplement the conventional peripheral blood progenitor cell transplant and should offset the generally observed post-transplant thrombocytopenia. To achieve this goal, we had proposed to first compare the effectiveness of GM-CSF and PIXY321 in priming megakaryocyte production in cultures of mononucleated and purified CD34+ cells from peripheral blood stem cell harvests. We also proposed to assess the results obtained in parallel to similar studies conducted with megakaryocyte progenitors from human bone marrow and fetal cord blood samples.

Summary of results of year 01: Midway through the first year, we substituted thrombopoietin (TPO), the purified growth factor mainly specific for the megakaryocyte lineage, for the aplastic serum mentioned in the original proposal. Cultures were carried out in serum-containing conditions, using autologous serum or plasma for peripheral blood progenitor cell (PB) cultures. Comparison of bone marrow (BM) and umbilical cord blood (UC) mononucleated cell cultures using aplastic serum indicated a higher megakaryocyte (MK) production from UC cultures. Mononucleated and CD34+ bone marrow cell cultures indicated that TPO/IL-3 was the most efficient cytokine combination for megakaryocyte production. Since most patients enrolled in the clinical trial comparing mobilization of GM-CSF vs. PIXY321 had low levels of CD34+ cells, cultures were carried out with mononucleated cells, and this study was started on year 01. The results indicated that in these conditions, the addition of IL-3 to TPO did not improve megakaryocytopoiesis.

Summary of results of year 02: In the process of optimizing megakaryocytopoiesis in BM, we discovered that a soluble factor from BM stroma enhanced the TPO-stimulated megakaryocyte production. These results were published in Stem Cells and a reprint was included in year 02 report (5). The study comparing the effectiveness of GM-CSF and PIXY321 in priming MK production in cultures of mononucleated cells from PB harvests was completed. The results described in detail in year 02 report show that the number of MK per CD34+ cells, was greater in PIXY321- than in GM-CSF-mobilized samples. However, since the frequency of CD34+ cells was greater in GM-CSF- than in PIXY321-mobilized samples, there was no significant difference in the overall absolute number of MK produced per mononucleated cell between PIXY321 and GM-CSF-mobilized samples. We are enclosing a reprint of our published findings (6). We also indicated that PIXY321 would not be further used since Immunex Corporation decided to cease developing this cytokine.

Summary of results of year 03: During year 03, we focused on: (a) further characterization of the BM stroma-enhancing effect on megakaryocytopoiesis, and (b) further characterization of megakaryocytopoiesis in BM, UC and PB, using exclusively CD34+ cells as seeding material, serum-free media and the usual cytokines as well as Promegapoietin, a novel chimeric protein that is a multifunctional dual agonist of c-mpl and human IL-3 receptors. In view of our results pointing to the beneficial effect of the TPO/IL-3 combination, it was imperative to investigate the effect of PMP on MK production. The methods of approach involved tissue culture and the effect of different factors on megakaryocyte development.

## BODY

Experimental Methods:

*1) Preparation of low density non-adherent mononuclear cells:* BM, UC and PB samples were collected in accordance with the guidelines of the Institutional Review Board on Human Subjects. BM, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in a special anticoagulant mixture designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin, 1 mM $Na_2EDTA$, 1 mM adenosine, 2 mM theophylline, 2.2 μM prostaglandin $E_1$ and 0.1 mg/ml DNase I. Marrow cells were repeatedly extracted from bone fragments with a modified MK medium (7) which consists of $Ca^{2+}$-$Mg^{2+}$-free phosphate-buffered saline (Dulbecco's PBS, Gibco) containing 13.6 mM Na citrate, 11 mM dextrose, 1 mM theophylline, 1 % bovine serum albumin, 2.2 μM $PGE_1$ and 0.1 mg/ml DNase I. Following homogenization by passage through a 18 gauge needle, low density cells were extracted with the use of Ficoll-Paque as described (8). Cells resuspended in MK medium were centrifuged at 380xg through a 10 % human serum albumin cushion in PBS to reduce platelet contamination. Residual red cells were lysed with $NH_4Cl$ as described (9) and the remaining cells recovered by centrifugation through a 10 % human serum albumin cushion. Adherent cells were discarded following overnight incubation in Iscove Modified Dulbecco's medium (IMDM) containing 10 % fetal bovine serum (FBS). All culture media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 4 μg/ml gentamycin, and incubation carried out at 37°C in a 5 % $CO_2$ fully humidified atmosphere.

UC was collected in the special anticoagulant. Buffy coat and red cells were submitted to Ficoll separation and the remaining steps carried out as for bone marrow cells. Frozen PB were rapidly thawed at 37°C and added to thawing medium consisting of IMDM, 25% FBS, 12.5 U/ml heparin and 1.25 mg% DNase I. Residual red cells were lyzed, and remaining MNCs were washed and treated as described above for the elimination of adherent cells.

*2) Purification of CD34+ cells:* CD34+ cells were purified by positive selection using the CD34 magnetic cell sorting Mini-MACS kit (Myltenyi Biotec, Auburn, CA) in accordance with the manufacturer's recommendation. A recovery of about 60 % CD34+ cells was obtained with a purity of 86.4 % ± 1.5 S.E.M (n=6) on the basis of flow cytometric analysis following staining with PE-anti-CD34 (HPCA-2).

*3) Bone marrow stroma:* A confluent stroma layer was prepared by adding to the adherent mononucleated cells a medium consisting of IMDM supplemented with 12.5% FBS, 12.5% horse serum, non-essential and essential aminoacids, Na pyruvate (100 mM), vitamins, hydrocortisone ($5 \times 10^{-6}$ M) and 2-mercaptoethanol ($10^{-8}$ M). At confluency, usually reached within a week, the adherent stromal cells were detached by adding trypsin (0.25%)-EDTA (1mM). Following washing with IMDM-base medium with 10% FBS, 200,000 or 800,000 cells/well were plated on 24- or 6-collagen-coated plates, respectively. The following day the adherent cells were irradiated with a dose of 12 Gy from a $^{137}Cs$ source (Gammacell 40, Nordion International Inc., Kanata, Ont., Canada). Collagen-coated plates were from Collaborative Research (Becton-Dickinson, San Jose, CA).

*4) Conditioned Media::* Conditioned media (CM) was prepared by adding IMDM containing 1% human serum albumin (HSA), 2.5% normal serum (NS) and 50 U/ml TPO (CM-TPO) to six wells-stroma-coated plates. After 48-72 hours supernatant was collected and centrifuged at 260xg for 10 min. at 4°C. In some experiments, CM was prepared without TPO.

*5) Culture conditions:* Low density non-adherent mononuclear cells and purified CD34+ cells were cultured for 12-14 days at 37°C at concentrations of $10^6$ and $5x10^4$ cells/ml, respectively, in an IMDM-based medium with 1 % human serum albumin and 2.5 % normal human serum which was found to be necessary for MK cultures. In order to prevent the inhibitory effects on MK growth of transforming growth factor-ß, ß-thromboglobulin and platelet factor 4 released from activated platelets (10-12), normal serum was obtained by recalcification of citrated platelet-free plasma. TPO (Zymogenetics Corp.) was used at a concentration of 50 U/ml (10 U = TPO quantity which stimulates one-half maximal proliferation of BaF3/mpl cells) which yielded the maximal concentration of MKs. Concentrations of IL-3 (R&D Systems, Minneapolis, MN), Stem Cell Factor (SCF) (R&D Systems, Minneapolis, MN) and Promegapoietin (PMP, Searle R&D) were 10 ng/ml (0.4-1.6 units based on 1 unit=ED50 of TF1 cell line proliferation), 50 ng/ml and 200 ng/ml, respectively, and were optimal for MK growth. The effect of stroma from human bone marrow was evaluated by culturing purified CD34+ cells in the presence or absence of TPO (Zymogenetics Corp., Seattle, WA) either in direct contact with stroma (contact) or in inserts (Collaborative Res., Becton Dickinson) separated from the stroma by a 0.41 µm pore membrane (non-contact). In control experiments CD34+ cells were cultured in inserts placed over stroma-free wells (stroma-free).

*6) Immunocytochemistry:* Adherent MK were scored following immunochemical staining. The well plates were washed twice with 0.05 M Tris, 0.15 M NaCl Ph 7.6 (TBS). After fixation for two minutes with methanol-acetone (1:1), sequential incubations, interspersed by washing with TBS, were carried out with blocking solution (5% human serum in TBS, 15 min), cocktail of MoAbs antiGPIIb (CD41) and GPIb (CD42b) (2 µg/ml,1 hour) and secondary antibody (rabbit anti-mouse IgG coupled to alkaline phosphatase - Sigma, diluted 1:200 in TBS-15% human serum, 30 min). Plates were then washed and incubated for 15 min with 0.1 M Tris pH 8.2, followed by 20 min incubation with alkaline phosphatase substrate Fast red (Sigma). The reaction was stopped by rinsing with water.

*7) Immunoenzymatic assays:* Stroma (confluency at 800,000 cells) in the absence or presence of TPO (50 U/ml), was incubated with IMDM-base medium containing 1 % HSA and 2.5 % NS for 12-14 days at 37°C in a 5 % $CO_2$ fully humidified atmosphere. The supernatant was then centrifuged to eliminate cell debris and samples were frozen (-70°C) until assayed. IL-6, IL-3 and SCF were measured using Elisa kits from R&D Systems in accordance with the manufacturer's recommendation. The control sample was IMDM-based medium with 1 %HSA and 2.5 %NS and the values of IL-6, IL-3 and SCF found were substracted from the sample values.

*8) Cell labeling and flow cytometric analysis:* The relative frequency of megakaryocyte progenitors was determined at day 1 on mononucleated and CD34+-enriched cells. Cell aliquots were treated with 200 pkat chymopapain (Knoll Pharm. Co., Lincolnshire, IL) This treatment detaches most platelets and platelet fragments from cells which otherwise would stain with the anti-CD41a antibody (anti-GPIIb/IIIa). After washing the cells were double-stained with phycoerythrin (PE) conjugated-anti-CD34 (HPCA-2, Becton-Dickinson) and fluorescein isothiocyanate (FITC) conjugated anti-CD41a (Immunotech-Amac, Westbrook, ME) and analyzed by flow cytometry. Negative controls were PE-anti-mouse $IgG_1$ and FITC-anti-mouse $IgG_1$ used at equivalent $IgG_1$ concentrations. The relative frequency of mature MKs was determined following 12-14 days culture by flow cytometric analysis of cells stained with FITC-anti-CD41a. Flow cytometric analysis was performed using a Coulter ELITE dual laser flow cytometer. Fluorescence attributable to FITC- and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm and adjusted to 0.3 W. Emission from fluorescein and PE was measured using band pass filters of 530 ± 15 nm and 575 ± 15 nm, respectively. The per cent positive cells was calculated by substracting the percent positive of the isotype control within the same integration region.

*9) Clonogenic Assay:* Clonogenic assays were carried out using a serum-free collagen assay [26] (Easyclone MegaClonogenic Assay, Hemeris) according to the manufacturer's instructions. The cytokines used were TPO, 50 U/ml, and/or IL-3, 10 ng/ml; or PMP, 200 ng/ml. CFU(colony-forming unit)-MK were scored after 8 days, and BFU (burst-forming unit)-MK after 14 days following fixation with methanol:acetone (1:1) and immunostaining with a-GPIb and a-GPIIb (Immunotech, Westbrook, ME). Antibody binding was revealed with an APAAP kit (Dako).

*10) Statistical analysis:* Comparisons between CD34+ cell sources, cytokines, and media were done using the two tailed student t-test. Samples within the same cell sources were compared using the paired t-test, and samples from different cell sources were compared using the unpaired t-test. Statistically significant results were those comparisons that had p-values less than 0.05.

Results:

*CHARACTERIZATION OF THE BONE MARROW STROMA ENHANCING ACTIVITY ON MEGAKARYOCYTE PRODUCTION*

*1) Role of stromal cytokines on MK production* : To evaluate whether the stromal enhancing activity on MK production was related to TPO release by stromal cells, we analyzed the effect of stroma on CD34+ cultures supplemented or not with exogenous TPO. The addition of TPO to CD34+ cells either in the presence or absence of stroma significantly stimulated MK development (table I). In the absence of exogenous TPO, MK formation ($2x10^3$) which was observed only in the presence of stroma, was decreased by 50 % following the addition of soluble c-Mpl receptor (10 µg/ml). The endogenous TPO, presumably responsible for this relatively small stroma-induced MK production, corresponded to a level of 0.8-1.5 U/ml TPO in our culture conditions. Such a small TPO concentration did not enhance MK production stimulated by 50 U/ml TPO in a stroma-free system.

We next evaluated the stromal release of different pleiotropic growth factors that are known to modulate MK development (5, 13-15) . The concentrations of IL-3, SCF and IL-6, released in the culture medium were not significantly different in the presence or absence of TPO ( 5.5±2, 962±423 and 306 ±113 pg/ml of IL-3, SCF and IL-6, respectively, without TPO and 4±1, 940±269, and 347±94 pg/ml of IL-3, SCF and IL-6, respectively with TPO). Addition of IL-3, IL-6 and SCF, in concentrations secreted by stroma, enhanced by 58 % TPO-stimulated MK production in stroma-free conditions ($3.8x10^5 \pm 4x10^4$ SEM MKs in presence of TPO/IL-3/SCF/IL-6 vs. $2.45\ x10^5 \pm 5.7x10^4$ SEM MKs in presence of TPO alone, n=4, $p < 0.02$).

The constitutive or inducible nature of the MK enhancing activity of stroma was investigated using conditioned medium. As can be seen in figure 1, a 6-fold stromal enhancing effect on MK development was obtained in the presence of stroma conditioned medium obtained either in the presence or absence of TPO.

*2) Role of heparan sulfates on MK production:* The effect of various TPO/heparan sulfate combinations on enhancement of MK production was investigated. Heparan sulfate (HS) from both bovine intestinal mucosa and bovine kidney as well as modified heparins (completely desulfated-N-acetylated heparin, completely desulfated-N-sulfated heparin and

N-desulfated-N-acetylated heparin) at concentrations ranging from 0.5 to 50 μg/ml did not have any enhancing effect on MK development stimulated by TPO and stromal concentrations of IL-3, IL-6 and SCF.

*3) Effect of direct contact of CD34+ cells with stroma on MK production:* Culture of CD34+ cells in the absence of stroma and TPO did not result in MK formation. In the presence of stroma, while TPO enhanced MK production, adherent (AMK), non adherent-MKs (NAMK) and proplatelet structures occurred whether in the presence or absence of TPO. In all cases, the majority of MKs (89 %) were in the non-adherent phase (table 2). Similar number of MKs were obtained in the presence of TPO whether non-contact or contact systems (AMK + NAMK) were used ($1.9x10^4 \pm 0.9x10^4$ SEM MKs in non-contact vs. $1.4x10^4 \pm 0.7x10^4$ SEM MKs in contact system, n=3, $p>0.1$).

*OPTIMIZED MEGAKARYOCYTE EXPANSION IN A SERUM-FREE MEDIUM*

CD34+ cells were used in our cultures in view of the recently reported undetectable levels of malignant cells in CD34+ cells from PB of patients with breast cancer, while leukaphereses products contained epithelial cells (16).

*1) Relative frequency of ex vivo expanded megakaryocytes:* For each hematopoietic source, no significant difference was found in the frequency of CD41+ cells produced by cultures supplemented with PMP when compared to those supplemented with either TPO alone, or TPO+IL-3 (Figure 2a and b). CD41+ cell frequencies were significantly greater in PMP, TPO, and TPO+IL-3-supplemented cultures when compared to IL-3-supplemented cultures. BM and UC consistently yielded significantly higher CD41+ frequencies than PB for all cytokine and culture medium combinations. BM CD34+ cells cultured in serum-free medium had a significantly higher CD41+ cell frequency when compared to cultures with serum for all cytokine combinations tested.

*2) Total megakaryocyte production:* UC produced significantly higher numbers of MKs than BM or PB under all conditions (Figures 3a and b). While the combination of TPO+IL-3 resulted in greater MK production than the addition of PMP in UC in serum-containing conditions, the reverse effect was obtained in serum-free conditions. Overall, using PMP resulted in significantly greater MK production than using TPO or IL-3. There was no significant difference between BM and PB cells cultured in serum-free medium. In serum-containing medium, BM produced significantly more MKs than PB. The addition of IL-3 to TPO synergistically increased MK production for BM and PB. Synergy was also observed with PMP. Compared to the activity of TPO and IL-3 added singly, synergistic increases in MK production were also seen when UC cells were cultured with PMP in MSFM and TPO+IL-3 in the presence serum. However, an additive effect was seen in UC cultures with TPO+IL-3 in MSFM and for PMP in serum-containing medium.

*3) Clonogenic capacity of CD34+ cells:* Similarly to liquid suspension systems, there were no significant differences between PMP and TPO+IL-3 in the number of either CFU-MK or BFU-MK colonies derived from any cell source (Fig. 4a). UC CD34+ cells produced significantly more CFU-MK and BFU-MK colonies than BM. PBPCs had the lowest clonogenic capacity (Figure 4a). The size of BFU-MKs from UC (>200 cells per colony) was larger than the equivalent colonies from BM or PBPCs (data not shown). When the number of colonies per seeded CD34+/CD41+ cell was calculated, UC cells produced a ratio of 3 BFU-MK colonies/MK progenitor, and 4.5 CFU-MK colonies/MK progenitor, significantly higher than that by BM or PB (Fig. 4b).

Conclusions: While the enhancing effect of a stromal soluble factor on MK production was confirmed with the use of conditioned medium, we were not able to identify this factor(s). The stromal enhancing activity is neither accounted for by stromal TPO, IL-3, IL-6, SCF and heparan sulfate activities, nor modified by physical contact. The main information gained from this investigation, relative to the overall objectives of our proposal, pertains to the possible use of autologous stromal conditioned medium to enhance MK production. Physical contact of MK progenitors with BM stroma is not necessary.

PMP (promegapoietin) induced an equivalent synergistic increase in MK production as TPO+IL-3 when compared to TPO or IL-3 alone in BM and PB. The megakaryocytopoietic activity of UC CD34+ cells with PMP and TPO+IL-3 was dependent on the culture medium. The effect of TPO+IL-3 was synergistic compared to TPO or IL-3 alone in the presence of serum, but additive in serum-free medium; the inverse was true for PMP. UC CD34+ cells were several times more proliferative than either BM or PB in liquid culture, resulting in much greater MK production. In a collagen-based serum-free clonogenic assay, UC produced significantly greater numbers and larger CFU-MK and BFU-MK colonies, both on an absolute basis, and also per CD34+/41+ cell. This was despite the fact that BM had around twice as many CD34+/41+ cells in the initial CD34+ cell population as compared to PB or UC. Thus PMP is the functional equivalent of TPO+IL-3 in inducing megakaryocytopoiesis; its activity on UC cells is dependent on the presence or absence of serum in the culture medium. In view of the FDA removal of IL-3 for clinical use, PMP appears to be the cytokine of choice to be considered for optimal MK production in *ex vivo* expansion protocols.

Our long-range goal, as described in our original proposal, is the clinical use of an *ex vivo* expanded-rich product. The rationale of using this material over administration of exogenous TPO is the extremely high levels of TPO in post-transplant breast cancer patients (17). This suggests that administration of supraphysiologic levels of TPO may not be useful. The first clinical experience with transfusion of *ex vivo* expanded MK was recently reported (18) and demonstrated the safety of the procedure in ten patients with breast cancer or lymphoma undergoing stem cell transplant. The two patients receiving the highest numbers of MKs did not require platelet transfusions, suggesting that this approach may have major clinical impact. In this study, however, it was not determined whether the MK administered were in a differentiation stage that was conducive to maximal platelet production. Moreover, small 25 $cm^2$ flasks were used, which required over 200 flasks for culturing large numbers of MK. We will use more efficient culture techniques in order to supplement the autologous PB transplant with autologous *ex vivo* expanded MK as an alternative to TPO administration.

During our fourth and last year of support, we shall optimize the *ex vivo* expansion of MKs using PMP, while taking advantage of a recent report describing 26-weeks expansion of MK progenitors upon using a distinct culture protocol and supplementing TPO with Flt-3-L (19).

## REFERENCES


1. Peters WP, Rosner G, Ross, M et al. Comparative effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony colony-stimulating factor (G-CSF) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. Blood 81:1709 (1993).

2. Takamatsu Y, Harada M, Teshima T et al. Relationship of infused CFU-GM and CFU-Mk mobilized by chemotherapy with or without G-CSF to platelet recovery after autologous blood stem cell transplantation. Exp Hematol. 23:8 (1995).

3. William SF, Bitran JD, Richards JM et al. Peripheral blood-derived stem cell collections for use in autologous transplantation after high dose chemotherapy: an alternative approach. Bone Marrow Transplant 5:129 (1990).

4. Kessinger A, Armitage JO, Landmark JM et al. Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. Blood 71:723 (1988).

5. Schattner, M, Lefebvre P, Spanier Mingolelli S, Goolsby CL, Rademaker A, White JG, Foster D, Green D, Cohen I. Thrombopoietin-stimulated ex vivo expansion of human bone marrow megakaryocytes. Stem Cells, 14:207 (1996).

6. Lefebvre P, Winter JN, Rademaker AW, Goolsby C, Cohen I. *In vitro* production of megakaryocytes from PIXY321 versus GM-CSF-mobilized peripheral blood progenitor cells. Stem Cells, 15:112 (1997).

7. Tomer A, Friese P, Conklin R. Flow cytometric analysis of megakaryocytes from patients with abnormal platelet counts. Blood 74:594 (1989).

8. Debili N, Hegyi E, Navarro S et al. *In vivo* effects of hematopoietic growth factors on the proliferation, endoreplication, and maturation of human megakaryocytes. Blood 77:2326 (1991).

9. Terstappen LWMM, Salford M, Loken MR. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. Leukemia 4:657 (1990).

10. Mitjavilla MT, Vinci G, Villeval JL. Human platelet alpha granules contain a nonspecific inhibitor of megakaryocyte colony formation: its relationshipl to type ß transforming growth factor (TGF-ß). J Cell. Physiol. 134:93 (1988).

11. Abgrall JF, Han ZC, Sensebe L et al. Inhibitory effect of highly purified platelet ß-thromboglobulin on in vitro human megakaryocyte colony formation. Exp Hematol. 19:202 (1991).

12. Gewirtz AM, Calabretta B, Rucinski B et al. Inhibition of human megakaryocytopoiesis in vitro by platelet factor 4 (PF4) and a synthetic C-terminal PF4 peptide. J Clin Invest. 83:1477 (1989).

13. Zeigler FC, de Sauvage F, Widmer HR et al. In vitro megakaryocytic and thrombopoietic activity of c-mpl ligand (TPO) on purified hematopoietic stem cells. Blood 84:4045 (1994).

14. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood 85:1331 (1985).

15. Banu N, Wang J, Deng B et al. Modulation of megakaryocytopoiesis by thrombopoietin: the c-Mpl ligand. Blood 86:1331 (1995).

16. Hohaus S, Pforsich M, Murea S et al. Immunomagnetic selection of CD34+ peripheral blood stem cells for autografting in patients with breast cancer. Brit J Haematol. 97:881 (1997)

17. Shimazaki, C, Inaba, T, Uchiyama, H et al. Serum thrombopoietin levels in patients undergoing autologous peripheral blood stem cell transplantation. Bone marrow Transplantation 19: 771 (1997).

18. Bertolini F, Battaglia M, Pedrazzoli P et al. Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral progenitor cell transplant recipients. 89:2679 (1997).

19. Piacibello W, Sanavio F, Garetto L et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 89:2644 (1997).

## APPENDICES

*TABLES AND FIGURES*

Table 1. EFFECT OF STROMA ON MK PRODUCTION

| STROMA-FREE | | STROMA | |
|---|---|---|---|
| -TPO | + TPO | - TPO | +TPO |
| 0 (n=9) | 240 ± 72 (n=15) | 2 ± 1.5 (n=8) | 768 ± 125 (n=14) |

70 x $10^3$ CD34+ cells/ml in a 3 ml final volume (50 U/ml TPO, when present) were seeded in the insert compartment of a 6-well plate. Absolute MK numbers (x$10^3$) calculated following flow cytometric analysis. Values represent means ± SEM of n separate experiments ($p < 0.001$ for paired stroma-free + TPO vs. stroma + TPO).

Table 2. ADHERENT AND NON-ADHERENT MK FORMATION IN PRESENCE OF STROMA

| | ADHERENT MK | NON-ADHERENT MK |
|---|---|---|
| --- | 0.18 (n=2) | 1.5 (n=2) |
| TPO (10 U/ml) | 3 ± 1.5 SEM (n=3) | 186 ± 90 SEM (n=3) |

40x$10^3$ CD34+ cells/well (6-well plates) were seeded in the presence or absence of TPO. Values represent means of MK numbers (x$10^3$).

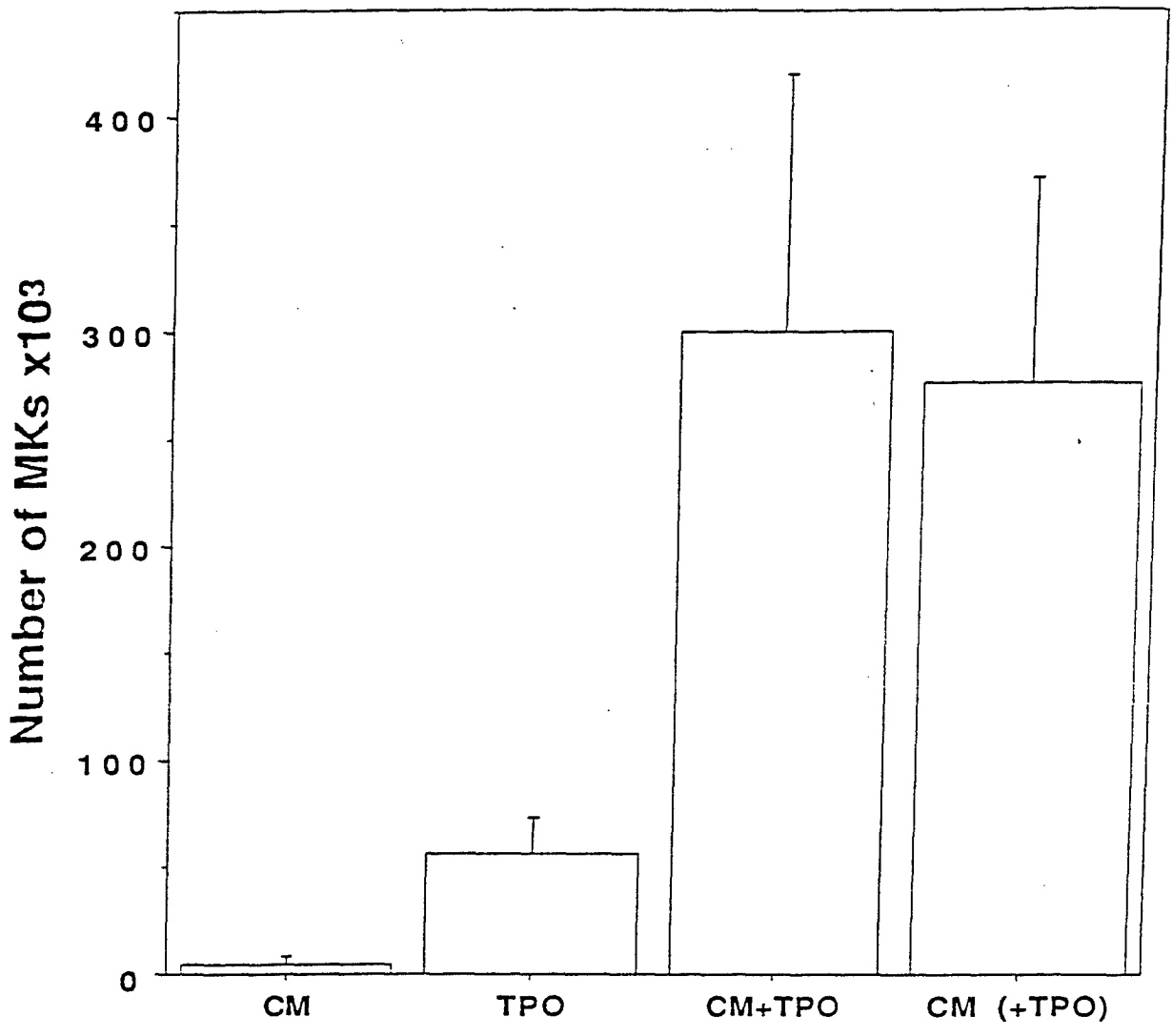


Fig. 1. Effect of stromal conditioned medium (CM) on MK production. $70x10^3$ $CD34^+$ cells/ml were seeded in a 2 ml final volume culture medium in the presence and absence of CM or TPO. CM+TPO, CM obtained in the presence of 50 U/ml TPO; CM(+TPO), CM obtained in the absence of TPO with 50 U/ml TPO added in culture with $CD34^+$ cells. The difference between MK numbers obtained with CM+TPO and CM(+TPO) was not statistically significant.

Figure 2a

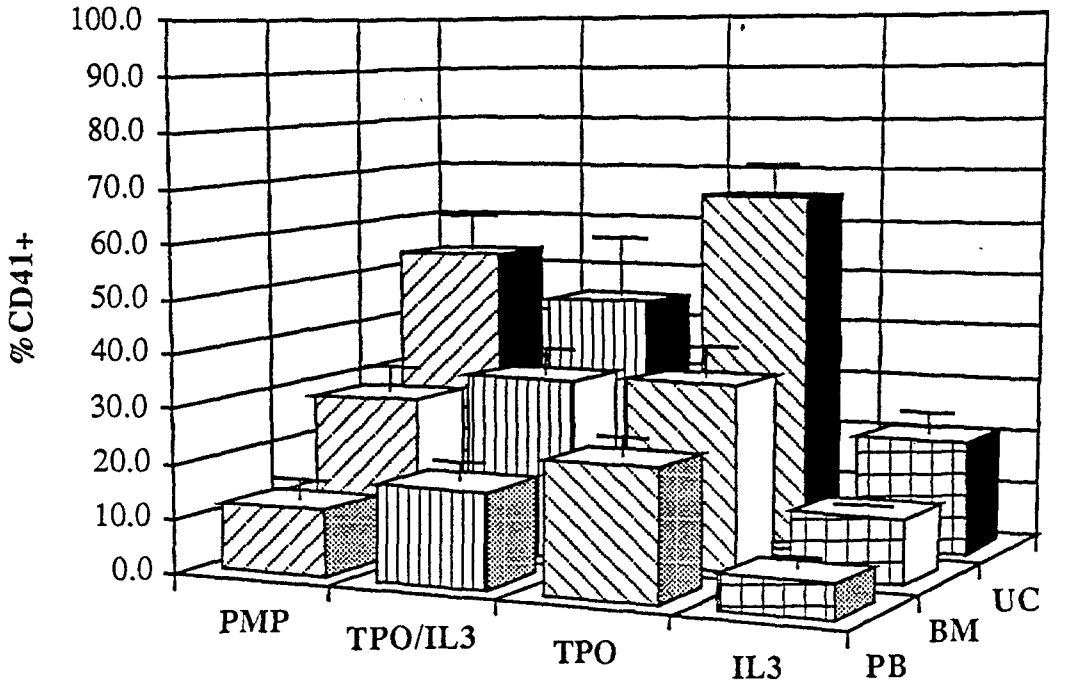


Figure 2b

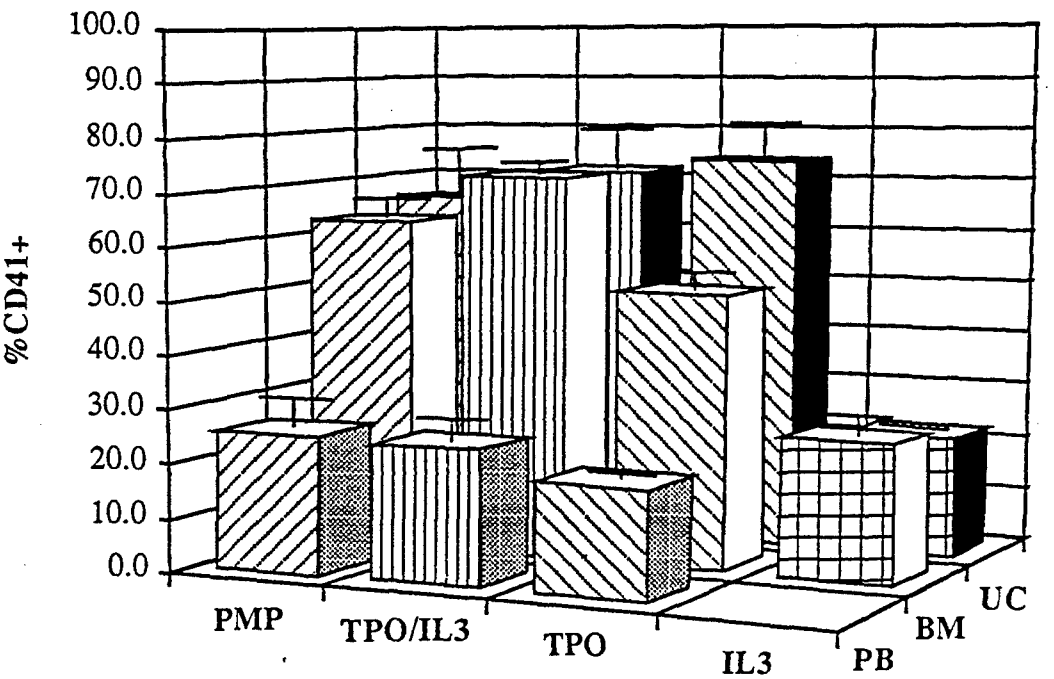


**Figure 2:** *Frequency of MKs found after 12 days of culture*
Mean percentage ± SEM of CD41+ cells found by flow ctometry in 6 BM, 3 UC and 3 PB samples cultured with the different cytokine combinations in either a) normal serum or b) MSFM.
a) UC had a significantly higher frequency than PB. UC samples treated with PMP or TPO alone had a significantly higher frequency than BM samples with the same cytokines.
b) BM and UC had a significantly higher frequency than PB. There was no significant difference between BM and UC except for the TPO-alone cultures, where UC had a significantly higher frequency.
There was no significant difference in the frequency of MKs found in either serum-supplemented or serum-free medium for UC or PB, but a significantly higher frequency of MKs was found in BM samples cultured in serum-free medium.

Figure 3a

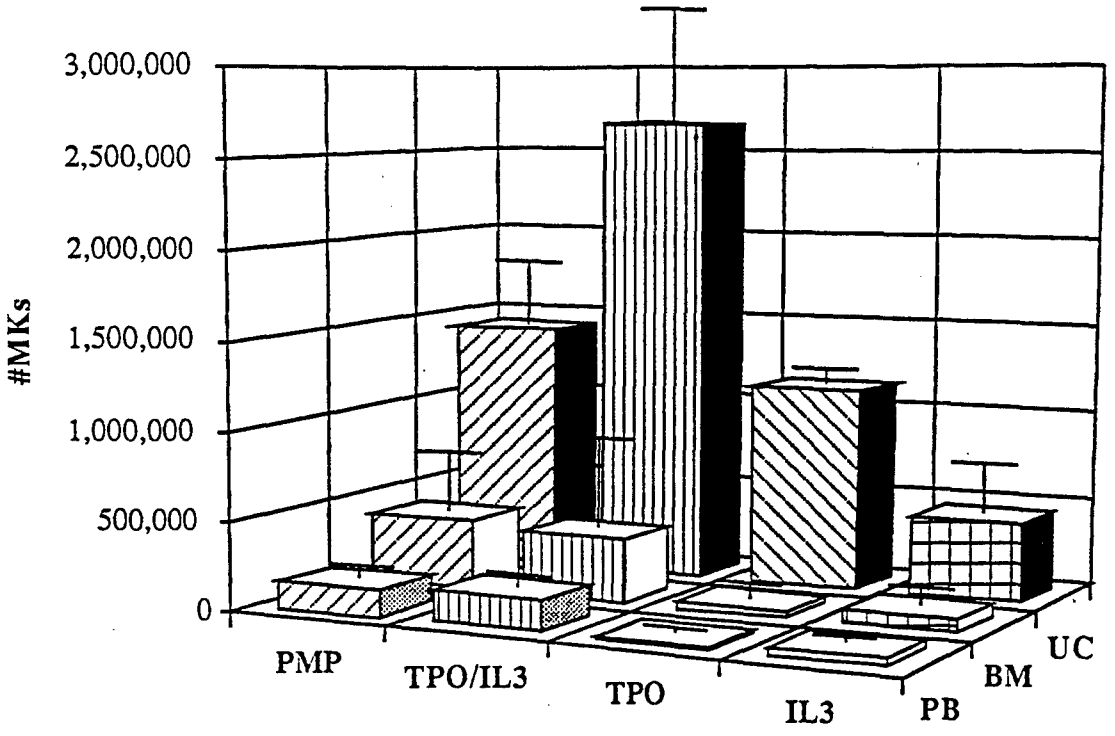


Figure 3b

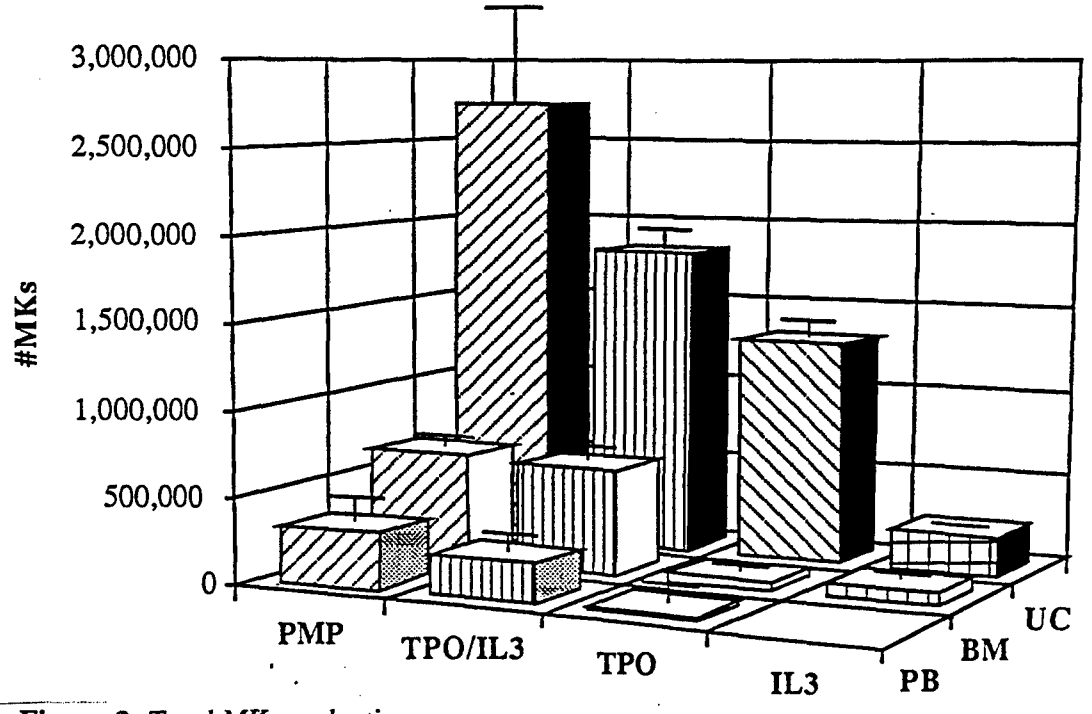


**Figure 3:** *Total MK production*
The number of megakaryocytes produced in each culture was calculated by multiplying the number of cells recovered from each sample by the frequency of MKs obtained in Figure 2 for both serum-supplemented (a) and serum-free cultures (b). The results are presented as the average ± SEM total number of MKs derived from an initial culture of 50,000 CD34 cells for each cell source and cytokine. UC (n=3) produced significantly more MKs than BM (n=6), which produced more MKs than PB (n=3), for all cytokine combinations tested.
No peripheral blood cultures were done with IL3 alone in the serum-free cultures. UC produced significantly more MKs than either BM or PB for all cytokine combinations tested. There were no significant differences between BM or PB.
There were no significant differences between the serum-supplemented and serum-free media in total MK production.

Figure 4a

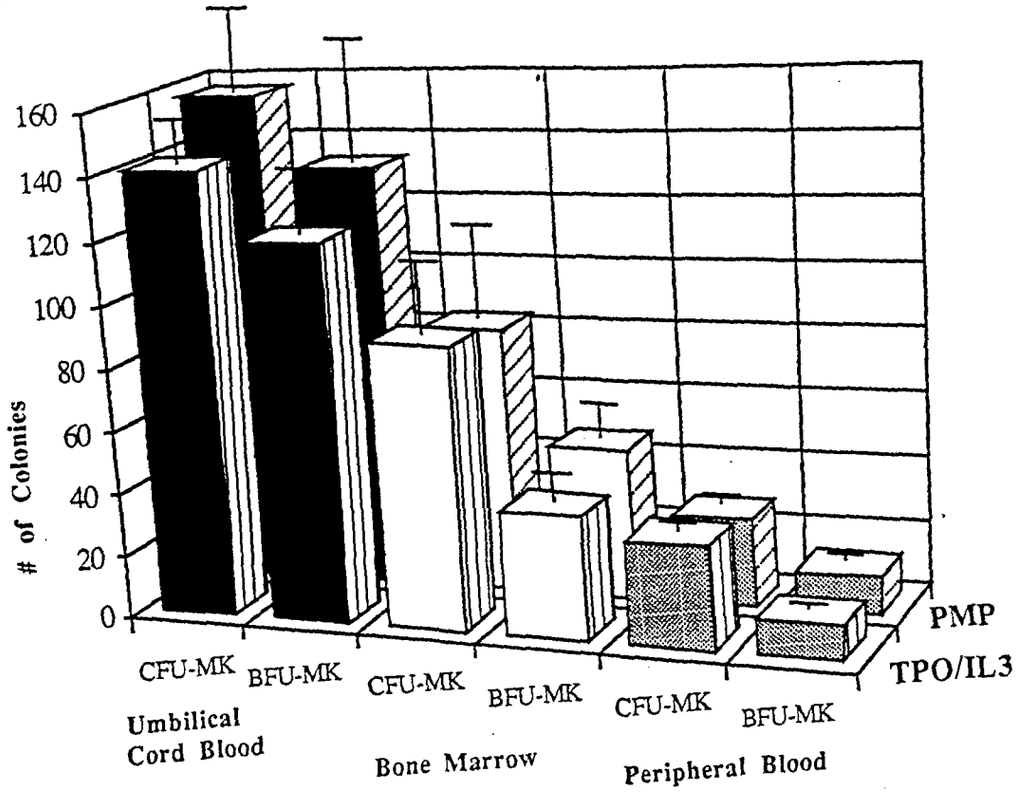


Figure 4b

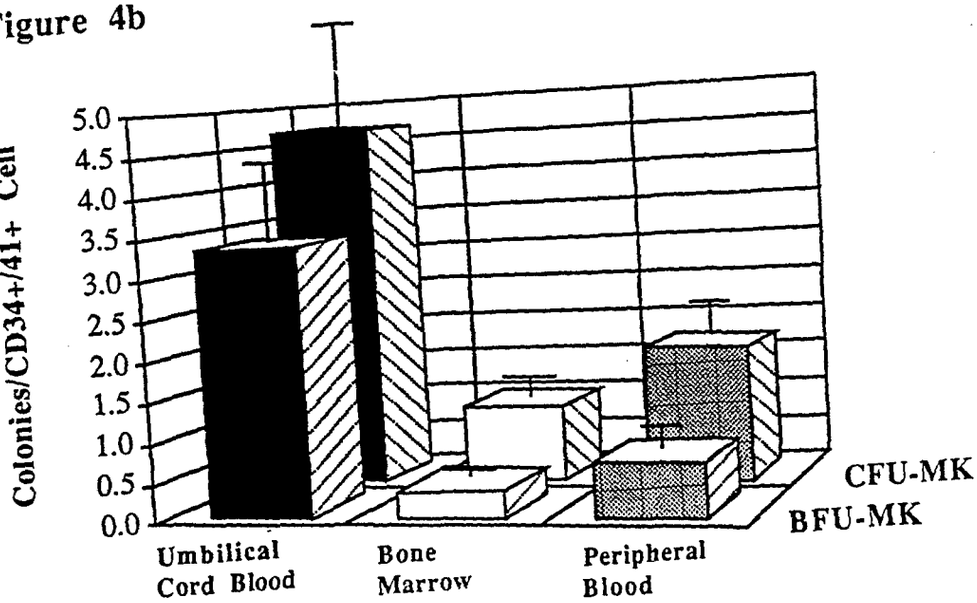


**Figure 4:** *Clonogenic capacity*
a) The mean number ± SEM of CFU-MK and BFU-MK for each source cultured with either PMP or TPO/IL3 is compared. For both CFU-MK and BFU-MK, UC produced significantly more colonies than BM ($p<0.05$), which produced significantly more than PB ($p=0.008$). There was no significant difference between PMP or TPO/IL3 in the number of colonies produced.
b) The number of colonies found in each clonogenic assay was divided by the number of CD34+/41+ cells in the seeded cell population. The average ± SEM for each cell source and colony type is represented. UC CD34/41 cells produced significantly more CFU-MK and BFU-MK than either BM ($p=0.017, 0.0005$) or PB ($p=0.046, 0.008$). There was no significant difference between BM or PB ($p=0.17$).

# In Vitro Production of Megakaryocytes from PIXY321 versus GM-CSF-Mobilized Peripheral Blood Progenitor Cells

P. LEFEBVRE,[a] J.N. WINTER,[b] A.W. RADEMAKER,[c] C. GOOLSBY,[d] I. COHEN[a]

[a]Department of Cell and Molecular Biology, [b]Department of Medicine, [c]Department of Preventive Medicine, [d]Department of Pathology and the Robert H. Lurie Cancer Center, Northwestern University Medical School, Chicago, Illinois, USA

Key Words. *Megakaryocytes · PIXY321 · GM-CSF · Thrombopoietin · Cyclophosphamide · Peripheral blood progenitor cells*

## ABSTRACT

**The generation of megakaryocytes (MK) from cultured peripheral blood progenitor cells (PBSC), harvested via apheresis, from 18 female breast cancer patients treated with either PIXY321 or GM-CSF was compared. Nonadherent mononuclear cells (MNC) were cultured in liquid suspension with 50 U/ml thrombopoietin (TPO) and 2.5% autologous heparinized plasma for 12 days. Flow cytometric analysis was used to measure the percentage of CD34⁺ on day 1 and CD41⁺ cells on day 12. The frequency of CD34⁺ cells was greater in GM-CSF-mobilized samples than in PIXY321-mobilized samples, and MK/MNC yields correlated directly with the number of CD34⁺ cells seeded. PIXY321-mobilized samples produced more MKs per CD34⁺ cell than GM-CSF-mobilized samples. Overall, there was no significant difference in the MK/MNC yield between PIXY321- and GM-CSF-mobilized samples. Cyclophosphamide (CY) increased the frequency of CD34⁺ cells and the corresponding MK/MNC yield for both cytokines, but had no effect on the MK/CD34⁺ yield. Compared to GM-CSF, PIXY321 mobilization resulted in increased CD34⁺ cell commitment to the MK lineage.** *Stem Cells 1997;15:112-118*

## INTRODUCTION

Treatment of metastatic breast cancer with high-dose chemotherapy is limited by myelosuppression and its complications. Reinfusion of cytokine-mobilized peripheral blood hematopoietic progenitors significantly shortens the period of neutropenia and thrombocytopenia when compared to conventional bone marrow autografts, although platelet engraftment may be delayed in comparison to neutrophil recovery [1-5].

PIXY321 is a recombinant chimeric polypeptide consisting of the binding and active domains of the cytokines GM-CSF and interleukin 3 (IL-3) [6], that has been shown to have enhanced stimulatory activity on megakaryocytopoiesis [7]. PIXY321 promotes more rapid platelet recovery in rhesus monkeys than GM-CSF and IL-3 combined [5], and has been shown to effectively mobilize peripheral blood progenitor cells (PBPC) in lymphoma patients [8]. Its capacity for ameliorating hematopoietic suppression has been the subject of recent clinical studies [9]. In phase I/II trials, PIXY321 administration has resulted in more rapid platelet recovery in patients receiving conventional-dose chemotherapy [10] and in those undergoing autologous bone marrow transplantation [11] when compared to historical controls. A recently reported prospective randomized trial, however, showed no advantage for PIXY321 over GM-CSF with regard to platelet nadirs or duration of thrombocytopenia in breast cancer patients receiving standard-dose chemotherapy [12].

Our goal is to develop megakaryocyte (MK) ex vivo expansion methods for clinical use. In view of the purported effects of PIXY321 on megakaryocytopoiesis, we hypothesized that PIXY321-mobilized PBPCs would have a greater capacity for generating MKs than GM-CSF-mobilized PBPCs. To test this hypothesis, nonadherent mononuclear cells (MNCs) from apheresis specimens of patients enrolled in a phase III randomized trial comparing PBPC mobilization by PIXY321 and GM-CSF [13] were cultured in the presence of thrombopoietin (TPO), the putative growth factor responsible for the proliferation and maturation of MKs [14-18]. The megakaryocytopoietic activity of the two populations was then measured and compared.

*Correspondence: Dr. Isaac Cohen, Northwestern University, RIC 1407, 345 East Superior Street, Chicago, IL 60611, USA.*
*Accepted for publication November 21, 1996.* 

## Materials and Methods

### Clinical Protocol

Women age 60 years or less, with advanced-stage breast cancer (either stage IV or IIIB, if inflammatory), were eligible for entry onto this multi-institutional clinical study. A total of 26 patients were randomized on this study, 18 at Northwestern Memorial Hospital, to receive either PIXY321 or GM-CSF to enhance the collection of PBPCs in the steady state and again following myelosuppressive chemotherapy (Fig. 1). This protocol was approved by the Northwestern University Institutional Review Board, and informed consent was obtained in all cases. Only those patients who were previously untreated for metastatic disease or who had received three or fewer cycles of chemotherapy for advanced disease were admitted to this trial. Women who had previously received adjuvant chemotherapy were eligible for study only if relapse had occurred six or more months after the completion of adjuvant treatment. Prior exposure to mitomycin C or carboplatin disqualified patients from entry. A minimum interval of three weeks from prior chemotherapy, irradiation, or surgery was required. Minimum hematologic parameters included an absolute neutrophil count of 1,500/μl, platelets ≥ $10^5$/ml, and hemoglobin ≥ 8 gm/dl. Induction therapy consisted of cyclophosphamide (CY) (2,400 mg/m²) and adriamycin (60 mg/m²). Patients who had not previously been treated for metastatic disease received three cycles, whereas those who had received one to three cycles of chemotherapy for advanced disease at the time of entry on study received only two. The last two patients from whom samples were obtained for this study of megakaryocytopoiesis received CY at 1,200 mg/m², because of concerns that the relatively poor mobilization observed on both arms was attributable to the high dose intensity. Taxol was substituted in two cases randomized to PIXY321 because of prior anthracycline exposure. Patients were then restaged, and only those who demonstrated a response to therapy went on to mobilization and stem cell transplant. Randomization was stratified according to the presence or absence of neoplastic cells in the bone marrow. After placement of an apheresis catheter, patients received either GM-CSF (Leukine®, Immunex Corp.; Seattle, WA), 375 μg/m² given once daily as a s.c. injection on days 1-7, or PIXY321 (Immunex), 375 μg/m² twice daily as a s.c. injection. Leukapheresis was performed on days 5, 6, and 7, using the CS-3000 Blood Cell Separator (Fenwal Division, Baxter Healthcare Corporation; Deerfield, IL). Aliquots of peripheral blood progenitor cells were stored in ampules containing $2 \times 10^8$ cells/ml, cryopreserved in a final concentration of 10% DMSO using a controlled rate freezer, and stored in vapor phase of liquid nitrogen. One week later, patients were treated with myelosuppressive doses of CY (4 gm/m²) followed by either PIXY321 or GM-CSF, as per the earlier randomization and at the same dose. PBPC collections began when the absolute neutrophil count exceeded 1,000/μl. Three sequential ten-liter collections were performed, and aliquots of the PBPCs were stored in ampules and cryopreserved as above for future study. Patients subsequently underwent high-dose chemotherapy with CY, thiotepa, and carboplatin, followed by stem cell reinfusion. Reinfusion was followed by either GM-CSF or PIXY321, according to a second randomization.

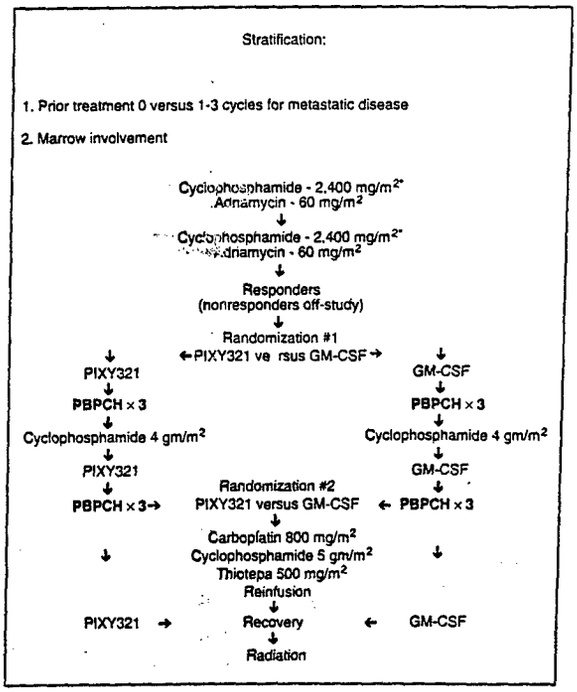


*Figure 1. Schema of clinical protocol. Patients previously treated for metastatic disease who had received up to three cycles of treatment for advanced disease at the time of study entry received two additional cycles of cyclophosphamide and adriamycin, whereas those who were untreated at study entry received three cycles. The peripheral blood progenitor cell harvest samples used for this study are highlighted in bold.*

**The last two patients from whom samples were obtained for this study received cyclophosphamide at 1,200 mg/m² because of concerns that the higher dose was sufficiently myelosuppressive to prevent mobilization; one was randomized to PIXY321 and one to GM-CSF.*

### Elimination of Adherent Cells

Frozen samples from all 18 Northwestern Memorial Hospital patients who underwent mobilization of PBPCs with either PIXY321 or GM-CSF, in the steady state and during recovery from myelosuppressive chemotherapy, were available for study. Ampules were thawed rapidly in a 37°C waterbath and transferred to a thawing medium of Iscove's modified Dulbecco's medium (IMDM; GIBCO;

Gaithersburg, MD) containing 20% fetal bovine serum (GIBCO), 12.5 μg/ml DNase I (Boehringer Mannheim; Indianapolis, IN), 12.5 U/ml heparin (GIBCO), 100 μM Dulbecco's modified Eagle's nonessential amino acids (NEA; GIBCO), 100 U/ml penicillin/100 μg/ml streptomycin (PS; Boehringer Mannheim) at 4°C and kept on ice for greater than 20 min. Erythrocytes were lysed by mixing the cell suspension 1:3 with 100 μM EDTA, 1 mM $KHCO_3$, 0.17 M $NH_4Cl$, pH 7.3, for 5 min [19]. This was underlaid with 5 ml 10% human serum albumin (HSA, Baxter Hyland; Deerfield, IL) in Dulbecco's phosphate-buffered solution (D-PBS; GIBCO), and centrifuged 6 min at 380 × *g*. The pellet was washed in 40 ml thawing medium and centrifuged 10 min at 260 × *g*. The cells were resuspended at $10^6$ cells/ml in 50% thawing medium/IMDM and incubated 2 hours or overnight at 37°C in 5% $CO_2$ in a Cellstar 2720 incubator (Queue Systems; Asheville, NC).

### Cell Culture

Nonadherent MNCs were collected and washed in 1% HSA/IMDM and resuspended at $10^6$ cells/ml in a culture medium of IMDM, PS, NEA, 1% HSA, 2.5% autologous plasma, 2 mM glutamine (Sigma; St. Louis, MO), and 50 U/ml TPO, where 10 U = quantity that stimulates one-half maximal proliferation of $BaF_3$/mpl cells (TPO; Zymogenetics; Seattle WA). The autologous plasma was made by collecting blood on 3.3 IU/ml preservative-free heparin (GIBCO) in siliconized tubes and centrifuging blood three times at 1,000 × *g*.

### Phenotypic Analysis

Viable cells were counted via the trypan blue exclusion method (samples were generally >85% viable) and up to 2.5 × $10^5$ cells were washed in special phosphate-buffered albumin containing 13.6 mM sodium citrate•$2H_2O$, 11 mM dextrose, 1 mM theophylline, 2.2 μM prostaglandin E1, 10% bovine serum albumin (Sigma) in D-PBS, pH 7.4. The cells were stained for 15 min on ice in the dark with either 1 μg phycoerythrin (PE)-conjugated-anti-CD34$^+$ (HPCA-2, Becton Dickinson; San Jose, CA) (day 1) or fluorescein isothiocyanate (FITC)-conjugated anti-CD41$^+$ (Immunotech/Amac; Westbrook, ME) (day 12). Corresponding negative controls were PE-anti-mouse $IgG_1$ (Becton Dickinson) and FITC-anti-mouse $IgG_1$ (Immunotech/Amac) used at equivalent $IgG_1$ concentrations. Cells were washed again in special phosphate-buffered albumin and fixed with 1% paraformaldehyde containing 0.067 M sodium cacodylate in saline. Flow cytometric analysis was performed using a Coulter Cytometry XL (Coulter Co.; Hialeah, FL) dual laser flow cytometer. Fluorescence attributable to FITC- and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm. Emission from FITC and PE was measured using band pass filters of 525 nm and 585 nm, respectively; 28,500 ± 11,900 total events were counted per sample. The integration region for the CD34$^+$ cells was set around the uppermost positive population of cells. The integration region for the CD41$^+$ cells was set at 1% positive in the negative population. The percent positive cells was calculated by subtracting the percent positive of the isotype control within the same integration region.

### Statistical Analysis

The mean and standard error of the mean were calculated for each parameter. The differences, after cytokine treatment and after cytokine-stimulated recovery from CY, in the percentage of CD34$^+$ cells in the MNC population and yields of MK per seeded MNC or CD34$^+$ cells within each cytokine, were compared using the two-tailed Wilcoxon signed rank test. Differences between the PIXY321 and GM-CSF populations across the same study conditions were analyzed using the two-tailed Mann-Whitney U rank sum test. These nonparametric tests were used due to the non-normal distribution of the data. Results of the MK/CD34$^+$ calculations after cytokine treatment and after cytokine-stimulated recovery from CY were pooled for each cytokine before analysis. The correlation of MK/MNC with the percentage of CD34$^+$ for each cytokine alone and when pooled was measured using the Spearman's rank correlation coefficient. Analysis was also done with outliers removed to assure that they did not significantly affect results. (Data not shown, as outliers did not affect statistical significance.)

## Results

### CD34$^+$ Cell Mobilization

Table 1 shows the clinical features of patients whose PBPCs were mobilized with either GM-CSF or PIXY321. There were no significant differences between the two arms with regard to sites of disease, prior treatment for advanced disease, and mean interval from chemotherapy to first dose of PIXY321 or GM-CSF (35 days versus 32 days). More patients on the GM-CSF arm had received adjuvant chemotherapy. While more adjuvant chemotherapy may have resulted in more cumulative myelosuppression, and thus poorer mobilization, this turned out not to be the case. When PBPCs were mobilized with cytokine alone, GM-CSF patients had a significantly higher percentage of CD34$^+$ cells in the nonadherent MNC population compared to PIXY321 patients (GM-CSF, 0.35% ± 0.09 versus PIXY321, 0.10% ± 0.014, $p = 0.006$). In apheresis products collected during cytokine-stimulated recovery from CY treatment, the mean percentage of CD34$^+$ cells in GM-CSF-mobilized MNC was three times higher than the mean for PIXY321-mobilized MNC, but did not

**Table 1.** Clinical features of patients

| Cytokine used | | PIXY321 | GM-CSF |
|---|---|---|---|
| Number of patients | | 10 | 8 |
| Age (median) | | 44 (Range: 33-57) | 39 (Range: 30-41) |
| Bone marrow involvement | | 10% | 12.5% |
| Prior treatment for advanced disease | | 50% | 50% |
| Prior adjuvant chemotherapy | | 30% | 75% |
| Prior radiation therapy | Chest wall | 10% | 25% |
| | Other | 0% | 12.5% |
| Total number of treatment cycles for advanced disease | Mean ± SE | 3.5 ± 0.2 | 3.8 ± 0.3 |
| | Median | 3.0 | 3.5 |
| Sites of disease | Bone | 40% | 63% |
| | Lung | 10% | 38% |
| | Lymph node | 40% | 12.5% |
| | Bone marrow | 10% | 12.5% |
| | Chest wall | 10% | 38% |
| Inflammatory breast cancer | | 20% | 12.5% |

reach statistical significance due to high variance (CY followed by GM-CSF, 1.61% ± 0.56 versus CY followed by PIXY321, 0.56% ± 0.15, $p$ = 0.08). Mobilization with myelosuppressive chemotherapy (CY) followed by cytokine produced a significantly higher percentage of CD34⁺ cells in the nonadherent MNC population in both patient groups (PIXY321 versus CY followed by PIXY321, $p$ = 0.006; GM-CSF versus CY followed by GM-CSF, $p$ = 0.008).

### Megakaryocytes per Mononuclear Cell Seeded

There were no significant differences between the MK/MNC yields of GM-CSF and PIXY321-mobilized patient samples, either when mobilized with cytokine alone (GM-CSF, 0.025 ± 0.007 versus PIXY321, 0.017 ± 0.003, $p$ = 0.56) or CY followed by cytokine (CY followed by GM-CSF, 0.197 ± 0.149 versus CY followed by PIXY321, 0.064 ± 0.018, $p$ = 0.61). MNCs collected during cytokine-stimulated recovery from CY produced significantly more MK/MNC than cytokine mobilization alone in every patient tested (PIXY321 versus CY followed by PIXY321, $p$ = 0.002; GM-CSF versus CY followed by GM-CSF, $p$ = 0.016).

### Correlation of MK/MNC with the Percentage of CD34⁺ Cells Seeded

The number of MK/MNC recovered at day 12 correlated directly with the percentage of CD34⁺ in the MNC at day 1 for both PIXY321- and GM-CSF-treated patients, both when mobilized with cytokine alone or CY followed by cytokine (Fig. 2). This was true whether the two groups were analyzed separately (PIXY321, $r$ = 0.69, $p$ < 0.001; GM-CSF, $r$ = 0.91, $p$ < 0.001), or combined (Fig. 2).

### Megakaryocytes per CD34⁺ Cell Seeded

The yield of MK per CD34⁺ cell seeded was calculated. There was no statistical difference in the yield of MK/CD34⁺

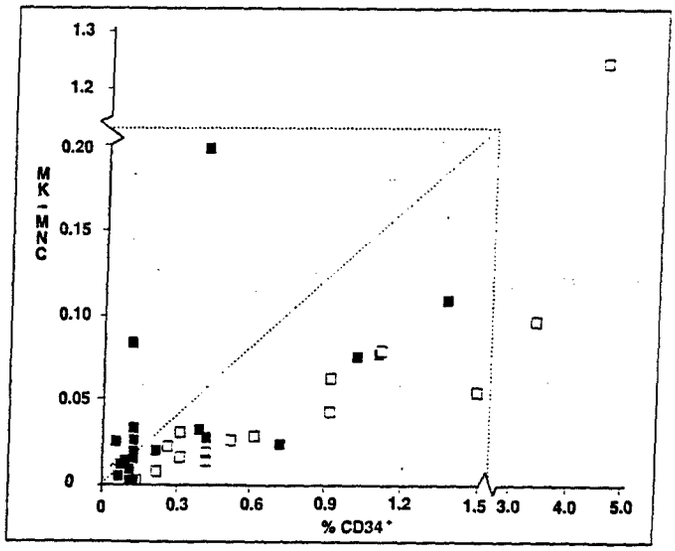


*Figure 2. Correlation of MK/MNC versus the percentage of CD34⁺. The number of megakaryocytes at day 12 per seeded nonadherent mononuclear cell on day 1 was correlated with the percentage of CD34⁺ cell in the nonadherent mononuclear cells on day 1 using the Spearman's rank analysis to take into account outlying data points. All PIXY321 (■) and GM-CSF (□) samples were combined. Graph was extended to show outliers. r = 0.70, p < 0.001.*

between PBPCs mobilized with cytokine alone and CY followed by cytokine (PIXY321, 23.0 ± 7.8 versus CY followed by PIXY321, 20.6 ± 8.3, $p = 0.32$; GM-CSF, 6.3 ± 2.8 versus CY followed by GM-CSF, 7.8 ± 2.7, $p = 0.99$). When the results for mobilization with cytokine alone and CY followed by cytokine were combined, PIXY321-mobilized PBPCs had a higher mean MK/CD34$^+$ cell seeded than GM-CSF-mobilized PBPCs (PIXY321 ± CY, 21.8 ± 5.53 versus GM-CSF ± CY, 8.37 ± 1.92, $p = 0.037$).

## Discussion

PIXY321 is being tested in clinical trials for its capacity to mobilize PBPCs and to alleviate post-transplant thrombocytopenia, based on the positive effects seen on megakaryocytopoiesis in vitro and platelet production in vivo. To determine if PIXY321-mobilized PBPCs produce more MKs ex vivo than standard treatment GM-CSF-mobilized PBPCs, we studied the production of MKs in liquid culture from apheresis samples obtained from patients receiving either PIXY321 or GM-CSF for the mobilization of PBPCs. The results of this study show that PIXY321-mobilized CD34$^+$ cells produced more MKs than GM-CSF-mobilized CD34$^+$ cells, while GM-CSF mobilized more CD34$^+$ cells per MNC collected than PIXY321. Together, this led to no net difference in the number of MKs produced per MNC cultured. One explanation for this finding may be that PIXY321 mobilizes a different subpopulation of CD34$^+$ cells than GM-CSF, with a greater commitment to the megakaryocytic lineage, but is a less effective mobilizer of hematopoietic progenitors overall.

In view of the small number of positive cells in certain samples, we ascertained the statistical validity of our results in three different ways. First, the CD34 frequencies were confirmed by another laboratory that analyzed the same patient samples for another study. While their sample preparation protocol was different from ours (in particular, they did not fractionate out adherent cells), their results and ours correlated to a significance of $p < 10^{-6}$. Second, the statistical method used was a nonparametric rank sum test, better suited to the non-normal distribution of the data. Samples were analyzed based on their relative rank to each other, not on their absolute values. By this method, the relative ranks of the samples would not be expected to change very much even if the absolute values were to change. Third, the statistical significance of the findings did not change when the absolute values were varied by their inherent uncertainty. Thus, we feel confident the results obtained are real.

*Bruno et al.* [7] first reported that human bone marrow cells cultured in vitro with PIXY321 sustained greater long-term megakaryocytopoiesis than GM-CSF alone. Our results indicate that PIXY321 exerts a similar effect ex vivo. The MK-promoting activity of PIXY321 may derive from the fact that it is a GM-CSF/IL-3 fusion protein. GM-CSF promotes the maturation of myeloid progenitors, making it effective in promoting neutrophil recovery [20, 21], whereas IL-3 promotes proliferation and differentiation of early progenitors, including those of the megakaryocytic lineage [22-24]. Clinically, IL-3 has been shown to induce a dose-dependent increase in thrombopoiesis in patients with normal bone marrow function and in patients with secondary bone marrow failure [25]. Taken together, these laboratory and clinical findings suggest that the IL-3 portion of PIXY321 could promote the mobilization of more MK progenitors compared to GM-CSF alone, and that culturing these progenitors in the presence of TPO would produce greater numbers of MKs [26]. Our results support this hypothesis.

There was no significant difference between PIXY321 and GM-CSF in the number of MKs generated per mononuclear cell. This was true whether PBPCs were mobilized by cytokine administration alone or by cytokine-stimulated recovery from myelosuppressive chemotherapy. If there is a relationship between PBPC mobilization and cytokine-stimulated progenitor recovery following chemotherapy, then these results may explain the recently reported observations of a phase III trial comparing PIXY321 and GM-CSF administration following conventional-dose chemotherapy for breast cancer [12]. No differences were found in platelet nadirs, duration of thrombocytopenia, or need for platelet transfusions. This may be explained by the fact that when compared to GM-CSF, PIXY321 is ineffective in mobilizing CD34$^+$ cells [13]. If the effect of PIXY321 on the kinetics of mobilization and recovery are the same, then even if PIXY321 enhances the proliferation of CD34$^+$ cells committed to the MK lineage compared to GM-CSF, the smaller pool of CD34$^+$ cells generated by PIXY321 would account for the lack of clinical impact seen in the randomized trial.

Given that MK progenitors express the CD34$^+$ marker, the relationship between the percentage of CD34$^+$ cells seeded and the MK/MNC produced by each sample was analyzed. As expected, the number of MK/MNC produced at day 12 correlated with the percentage of CD34$^+$ cells in the MNC culture on day 1, regardless of the cytokine used for mobilization. This suggests that, in general, the number of CD34$^+$ cells will determine the yield of MKs in ex vivo expansion. The clinical correlate of this finding was shown in the studies of *Takamatsu* [4], which showed that platelet recovery after autologous PBPC transplantation could be predicted accurately by the number of colony-forming units-granulocyte/macrophage ([CFU-GM], which in most studies correlates with CD34$^+$ cell content) infused, and that the number of CFU-GM in the autograft was equal to CFU-MK in predicting time-to-platelet recovery.

Myelosuppressive doses of CY followed by cytokine mobilized more CD34+ cells per MNC than the same cytokine alone. However, there was no significant difference seen in the generation of MKs per CD34+ cell. This shows that CD34+ cells mobilized by cytokine alone are similar to those mobilized during cytokine stimulated recovery from CY in their capacity to generate MKs. Myelosuppressive doses of CY have been shown to mobilize greater numbers of PBPCs [27, 28], and these are believed to be equivalent to bone marrow cells immunologically and in their in vitro growth characteristics [29]. Our results confirm these observations.

## Conclusion

This study shows that there is a difference in the type of CD34+ cell mobilized by PIXY321 compared to GM-CSF: PIXY321 mobilizes more cells committed to the MK lineage. Successful ex vivo expansion of MKs will depend on both the total number of CD34+ cells available (in this case, enhanced by GM-CSF), and their capacity for generating MKs. Therefore, the particular cytokine or cytokines used for mobilization may have profound effects on the results of an ex vivo expansion protocol.

## Acknowledgments

The authors would like to thank *Dr. Don Foster* of Zymogenetics (Seattle, WA) for supplying the TPO, *Drs. Leslie Garrison* and *David Green* for reviewing the manuscript, and *Vicki Mossiman* for assistance with flow cytometry.

This study was supported by United States Army Medical Research Acquisition Activity grant DAMD17-94-J-4465.

## References

1 Kessinger A, Armitage JO, Landmark JM et al. Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. Blood 1988;71:723-727.

2 Williams SF, Bitran JD, Richards JM et al. Peripheral blood-derived stem cell collections for use in autologous transplantation after high dose chemotherapy: an alternative approach. Bone Marrow Transplant 1990;5:129-133.

3 Peters WP, Rosner G, Ross M et al. Comparative effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. Blood 1993;81:1709-1719.

4 Takamatsu Y, Harada M, Teshima T et al. Relationship of infused CFU-GM and CFU-MK mobilized by chemotherapy with or without G-CSF to platelet recovery after autologous blood stem cell transplantation. Exp Hematol 1995;23:8-13.

5 Williams DE, Dunn JT, Park LS et al. A GM-CSF/IL-3 fusion protein promotes neutrophil and platelet recovery in sublethally irradiated rhesus monkeys. Biotechnol Ther 1993;4:17-29.

6 Curtis BM, Williams DE, Broxmeyer HE et al. Enhanced hematopoietic activity of a human granulocyte/macrophage colony-stimulating factor-interleukin 3 fusion protein. Proc Natl Acad Sci USA 1991;88:5809-5813.

7 Bruno E, Briddell RA, Cooper RJ et al. Recombinant GM-CSF/IL-3 fusion protein: its effect on in vitro human megakaryocytopoiesis. Exp Hematol 1992;20:494-499.

8 Bishop MR, O'Kane-Murphy BO, Jackson JD et al. Mobilization of blood stem cells with PIXY321: results of a Phase I trial. Blood 1995;86(suppl 1):579a.

9 Vadhan-Raj S. PIXY321 (GM-CSF/IL-3 fusion protein): biology and early clinical development. Stem Cells 1994;12:253-261.

10 Vadhan-Raj S, Papadopoulos NE, Burgess MA et al. Effects of PIXY321, a granulocyte-macrophage colony-stimulating factor/interleukin-3 fusion protein, on chemotherapy-induced multilineage myelosuppression in patients with sarcoma. J Clin Oncol 1994;12:715-724.

11 Vose JM, Anderson JE, Bierman PJ et al. Phase I/II trial of PIXY321 to enhance engraftment following autologous bone marrow transplantation for lymphoid malignancy. J Clin Oncol 1996;14:520-526.

12 O'Shaughnessy JA, Tolcher A, Riseberg D et al. Prospective, randomized trial of 5-fluorouracil, leucovorin, doxorubicin, and cyclophosphamide chemotherapy in combination with the interleukin-3/granulocyte-macrophage colony-stimulating factor (GM-CSF) fusion protein (PIXY321) versus GM-CSF in patients with advanced breast cancer. Blood 1996;87:2205-2211.

13 Winter JN, Lazarus HM, Rademaker AW et al. Comparison of PIXY321 and GM-CSF for mobilization of peripheral blood progenitor cells (PBPC) in advanced stage breast cancer. Blood 1995;86(suppl 1):578a.

14 de Sauvage FJ, Hass PE, Spencer SD et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature 1995;369:533-538.

15 Bartley TD, Bogenberger J, Hunt P et al. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 1994;77:1117-1124.

16 Lok S, Kaushansky K, Holly RD et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature 1994;369:565-568.

17 Kuter DJ, Beeler DL, Rosenberg RD. The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production. Proc Natl Acad Sci USA 1994;91:11104-11108.

18 Kato T, Iwamatsu A, Shimada Y et al. Purification and characterization of thrombopoietin derived from thrombocytopenic rat plasma. Blood 1994;84(suppl 1):329a.

19 Terstappen LW, Safford M, Loken MR. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. Leukemia 1990;4:657-663.

20 Nemunaitis J, Rabinowe SN, Singer JW et al. Recombinant granulocyte-macrophage colony-stimulating factor after autologous bone marrow transplantation for lymphoid cancer. N Engl J Med 1991;324:1773-1778.

21 Gerhartz HH, Engelhard M, Meusers P et al. Randomized, double-blind, placebo-controlled, Phase III study of recombinant human granulocyte-macrophage colony-stimulating factor as adjunct to induction treatment of high-grade malignant non-Hodgkin's lymphomas. Blood 1993;82:2329-2339.

22 Bruno E, Briddell RA, Hoffman R. Effect of recombinant and purified human hematopoietic growth factors on human megakaryocyte colony formation. Exp Hematol 1988;16:371-377.

23 Lu L, Briddell RA, Graham CD et al. Effect of recombinant and purified human hematopoietic growth factors on in vitro colony formation by enriched populations of human megakaryocyte progenitor cells. Br J Haematol 1988;70:149-156.

24 Teramura M, Katahira J, Hoshino S et al. Clonal growth of human megakaryocyte progenitors in serum-free cultures: effect of recombinant interleukin-3. Exp Hematol 1988;16:843-848.

25 Ganser A, Lindemann A, Seipelt G et al. Effect of recombinant human interleukin-3 in patients with normal hematopoiesis and in patients with bone marrow failure. Blood 1990;76:666-676.

26 Debili N, Wendling F, Katz A et al. The mpl-ligand or thrombopoietin or megakaryocyte growth and development factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors. Blood 1995;86:2516-2525.

27 To LB, Shepperd KM, Haylock DN et al. Single high doses of cyclophosphamide enable the collection of high numbers of hemopoietic stem cells from the peripheral blood. Exp Hematol 1990;18:442-447.

28 Tarella C, Ferrero D, Bregni M et al. Peripheral blood expansion of early progenitor cells after high-dose cyclophosphamide and rhGM-CSF. Eur J Cancer 1991;27:22-27.

29 Siena S, Bregni M, Brando B et al. Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. Blood 1989;74:1905-1914.

Recd 1/11/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Grants. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

94 J. 4465 AD-B233 503

Completed 1-13-00 a