Frequent Harvesting from Perfused Bone Marrow Cultures Results in Increased Overall Cell and Progenitor Expansion

Duk Jae Oh, Manfred R. Koller, and Bernhard O. Palsson*
Department of Chemical Engineering, University of Michigan, Ann Arbor, MI 48109-2136

Received December 6, 1993/Accepted April 6, 1994

The establishment of prolific long-term human bone marrow cultures has led to the development of hematopoietic bioreactor systems. A single batch expansion of bone marrow mononuclear cell populations leads to a 10- to 30-fold increase in total cell number and in the number of colony forming units-granulocyte/macrophage (CFU-GMs), and a four- to tenfold increase in the number of long-term culture initiating cells (LTC-ICs). In principle, unlimited expansion of cells should be attainable from a pool of stem cells if all the necessary requirements leading to stem cell maintenance and division are met. In this article, we take the first step toward the identification of factors that limit single batch expansion of ex vivo bone marrow cells in perfusion-based bioreactor systems. One possible constraint is the size of the growth surface area required. This constraint can be overcome by harvesting half the cell population periodically. We found that harvesting cells every 3 to 4 days, beginning on day 11 of culture, led to an extended growth period. Overall calculated cell expansion exceeded 100-fold and the CFU-GM expansion exceeded 30-fold over a 27-day period. These calculated values are based on growth that could be obtained from the harvested cell population. Growth of the adherent cell layer was stable, whereas the nonadherent cell population diminished with increasing number of passages. These results show that the bioreactor protocols published to date are suboptimal for long-term cultivation, and that further definition and refinement is likely to lead to even greater expansion of hematopoietic cell populations obtained from bone marrow. More importantly, these results show that the LTC-IC measured during the single pass expansion do have further expansion potential that can be realized by frequent harvesting. Finally, the present culture conditions provide a basis for an assay system for the identification of the factors that determine the long-term maintenance and replication of human stem cells ex vivo.

Key words: stem cells • bone marrow • ex vivo expansion • perfusion culture • hematopoiesis

INTRODUCTION

A major challenge that faces the tissue engineering community is to expand primary human cells ex vivo into clinically meaningful numbers for transplantation purposes. For bone marrow this challenge is particularly pressing given the rapid rise in the use of bone marrow transplantation (e.g., see recent review[4]). The goal is to generate an engrafting dose of cells from a small bone marrow aspirate.

Recently, the limitations of human Dexter cultures (see reviews in refs. 1 and 3) have been overcome by recognizing that the medium exchange rate was inadequate in the original protocol.[8,9] Subsequently, perfusion chambers have been developed for the growth of adult human bone marrow.[7] The more prolific cells obtained from cord blood have also been reported to grow in perfusion-based bioreactors.[5] Perfusion cultures of adult bone marrow result in 10- to 15-fold expansion of the total cell population[6,7] to yield cell densities exceeding 3×10^6 cells/cm^2. These chambers expand granulocyte-macrophage progenitor cells (CFU-GMs) by 10- to 30-fold. These perfusion bioreactors have also been shown to expand long-term culture-initiating cells (LTC-ICs).[6] Thus, the experimental data suggest that stem cell replication takes place in these cultures during single batch expansion. However, because cell densities are restricted to about 3 to 4×10^6 cells/cm^2 in the bioreactors, the total cell number obtainable from a single batch culture is limited. If this limitation can be overcome, sustained replication and maintenance of the stem cell compartment may be possible over an extended period of time.

The factors that constrain the expansion of cell numbers in these bioreactors are not known at present. Growth factor or oxygen delivery could be limited, or cell expansion could simply be restricted by the space available for growth. Here we address the latter possibility. We present a study demonstrating that extended growth of human bone marrow mononuclear cells (MNCs) obtained from adult donors can be obtained by increasing the cell growth area over time.

MATERIALS AND METHODS

Cells

Human bone marrow cells were obtained from heparinized aspirates from the iliac crest of informed and consenting individuals. The bone marrow was separated by a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the low density MNCs were collected and washed three times with Iscove's modified Dulbecco's me-

* To whom all correspondence should be addressed.

CCC 0006-3592/94/050609-08

dium (IMDM, Gibco, Grand Island, NY) as described previously.[6,7]

Culture Medium and Hematopoietic Growth Factors

Cell culture medium was IMDM containing 10% fetal bovine serum (Gibco), 10% horse serum (Gibco), 1% penicillin/streptomycin (Sigma, St. Louis, MO; 10,000 U/mL penicillin G and 10 mg/mL streptomycin), and 36 m*M* sodium bicarbonate (Sigma). The medium was supplemented with four growth factors: 2 ng/mL interleukin-3 (IL-3, R&D Systems, Minneapolis, MN), 5 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF, Immunex, Seattle, WA), 0.1 U/mL erythropoietin (Epo, Amgen, Thousand Oaks, CA), and 10 ng/mL mast cell growth factor (MGF, c-kit ligand, stem cell factor, Genzyme, Cambridge, MA).

Perfusion Systems

Two perfusion systems were utilized for bone marrow expansions. The first perfusion bioreactor system used has been described previously.[7] In the second perfusion system, 25-cm^2 tissue-culture-treated T-flasks (Costar, Cambridge, MA) replaced the reactor part of the system described.[7] Flasks were modified to have two ports for medium inlet and outlet. The depth of medium in the flask was fixed by the height of outlet port location. The working volume per flask could not be controlled accurately, but was in the range of 6 to 8 mL. The fresh medium was supplied from a syringe pump at a nominal rate of 5.64 mL/day and the spent medium was collected in a waste bottle using a peristaltic pump (Masterflex, Chicago, IL). Exit flow rates were difficult to control accurately and the flow rate fluctuated over time.

When the operation was terminated, nonadherent cells were harvested by first collecting the cell suspension and then by washing twice with Hank's balanced salt solution. These fractions were pooled and labeled as nonadherent cells. Adherent cells were collected following trypsinization for 20 min and by washing once with growth medium. Harvested cells were counted and analyzed for progenitor cell densities.

Partial Harvest of Nonadherent or Total Cells

In partial harvest of nonadherent cells, a fraction (50%, 70%, or 90%) of nonadherent cells in the perfusion bioreactor were sampled once during the culture period, and the cells remaining in the bioreactor were returned for continued growth. In order to harvest nonadherent cells, a small air bubble was introduced, and the bioreactor was gently shaken to obtain the nonadherent cells in suspension that could then be removed by medium withdrawal. This sampling procedure was cumbersome, so we used perfused T-flasks for subsequent frequent harvesting protocols. One half of the cells cultured in perfused T-flasks were repeatedly harvested over time (every 2 to 4 days).

For further fractionation of the adherent cell population, the adherent cells were resuspended following centrifugation in culture medium and incubated in a T-flask. Because most of the stromal cells were strongly adherent after 1 h of incubation, nonstromal cells in the nonadherent cell population were then collected. In order to compare the performance of the repeated harvest protocol with controls, which were untouched during the culture period, or with controls in which 100% of the cells were returned, cell and total CFU-GM numbers were calculated based on the cell numbers that can be obtained from both the returned and the removed cells, assuming that the latter had also been plated and grown.

Cell Count and Colony Assays

The numbers of MNCs were measured by an electronic cell counter (Coulter Electronics, Hialeah, FL) using a filtered cetrimide aqueous solution containing 30 g/L cetrimide (hexadecyl trimethylammonium bromide, Sigma), 8.3 g/L NaCl (Baker Co., Phillipsburg, NJ), and 0.37 g/L EDTA (ethylenediaminetetraacetic acid, Sigma). Clonogenic cells from the inoculum and harvested cells were enumerated. The cells were suspended at a density of 2×10^4 cells/mL in methylcellulose (Terry Fox Labs, Vancouver, BC, Canada) containing 2 U/mL Epo, 2 ng/mL IL-3, 5 ng/mL GM-CSF, and 5 ng/mL G-CSF (granulocyte colony stimulating factor; Neupogen, Amgen). After 2 weeks in a humidified 5% O_2 and 5% CO_2 incubator, cell colonies comprising more than 50 cells in the methylcellulose were counted.

RESULTS

The expansion of adult human bone marrow progenitor cells and MNCs in perfusion bioreactors has been recently reported.[6,7] These reports describe growth of an MNC population during a single batch expansion in which no cells were removed during the growth period. Here we wish to investigate whether the space for cell growth is limiting the cell expansion obtained under perfusion conditions.

Table I. Total cell expansions in bioreactors untouched and sampled once.

Culture time (day)	Untouched control (10^6 cells/reactor)	Sampled once (10^6 cells/reactor)
0 (inoculum)	4.0	4.0
13[b]	—	5.15 ± 1.20[a]
16	44.13 ± 7.00[a]	—
17	—	40.43 ± 2.24[a]
Expansion (total)	11.03 ± 1.75[a]	11.40 ± 0.86[a]

[a]Mean ± S.D. of three data points.
[b]Fifty percent of nonadherent cells were removed.

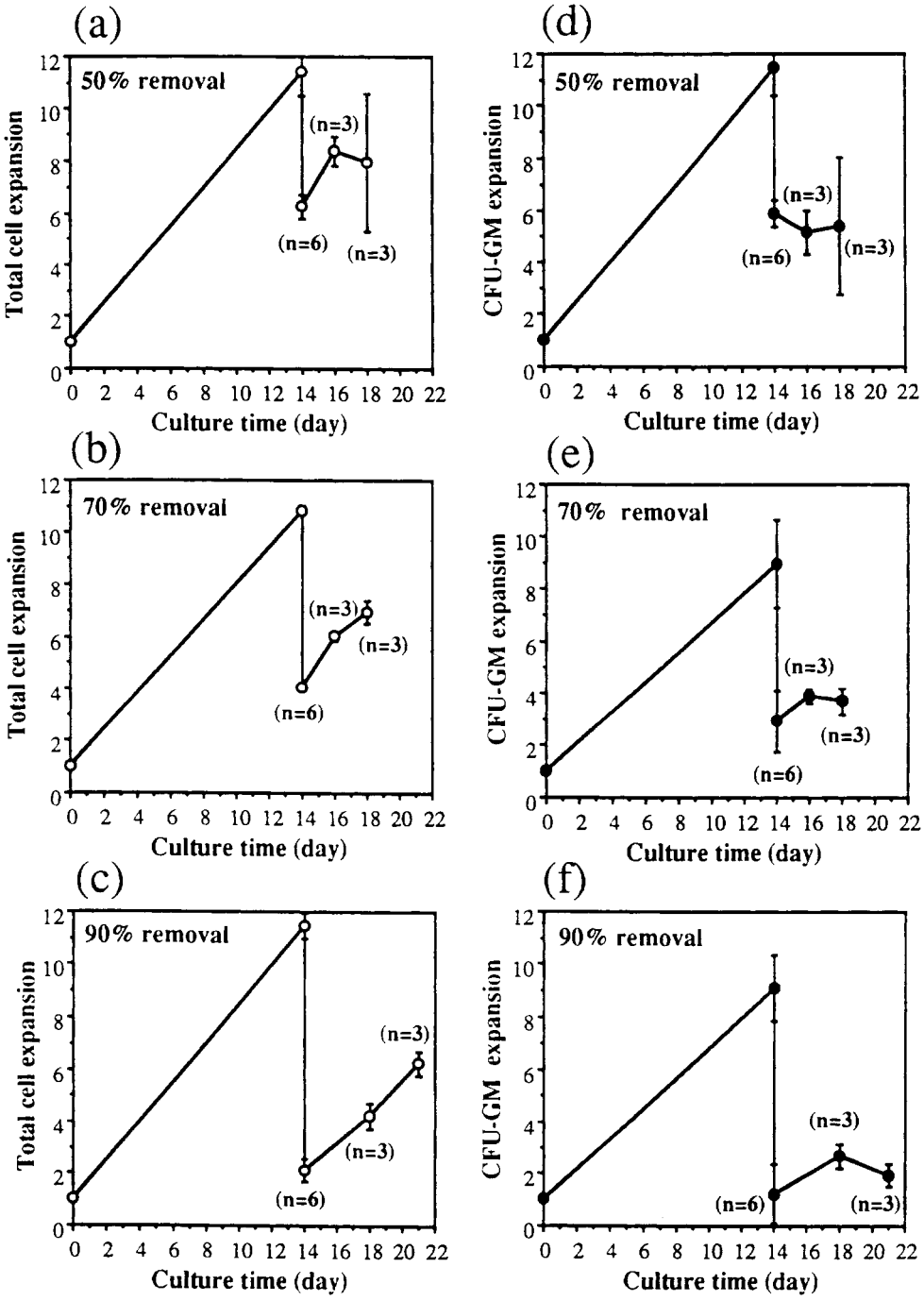

Figure 1. Total cell and CFU-GM expansion in bioreactors that were sampled once on day 14. Four million cells were inoculated into each bioreactor and nonadherent cells were sampled at day 14 in different fractions (a and d: 50%, b and e: 70%, c and f: 90%). Error bars show ± SD of (n) reactors sampled.

Our first experiment was to sample cells at one time point during the cultivation period in the bioreactors and determine if there were any detrimental effects on cell expansion. We thus removed 50% of the nonadherent cells after 13 days of culture and allowed the remaining cells to continue to grow. The results show that about the same total number of cells was obtained as from untreated control bioreactor cultures (Table I). This result suggests that the potential for expansion in the cell population is greater than allowed for by a single batch cultivation. The cells removed on day 13 potentially could have been cultivated further in a separate bioreactor. However, adherent cells were not removed and therefore we expect that the growth of the removed cells alone would differ from those that are left in the perfusion bioreactor.

The next question is: Is the cell growth limited in the chambers by "crowding"? If the answer is yes, then the cell population that reaches saturation at approximately day

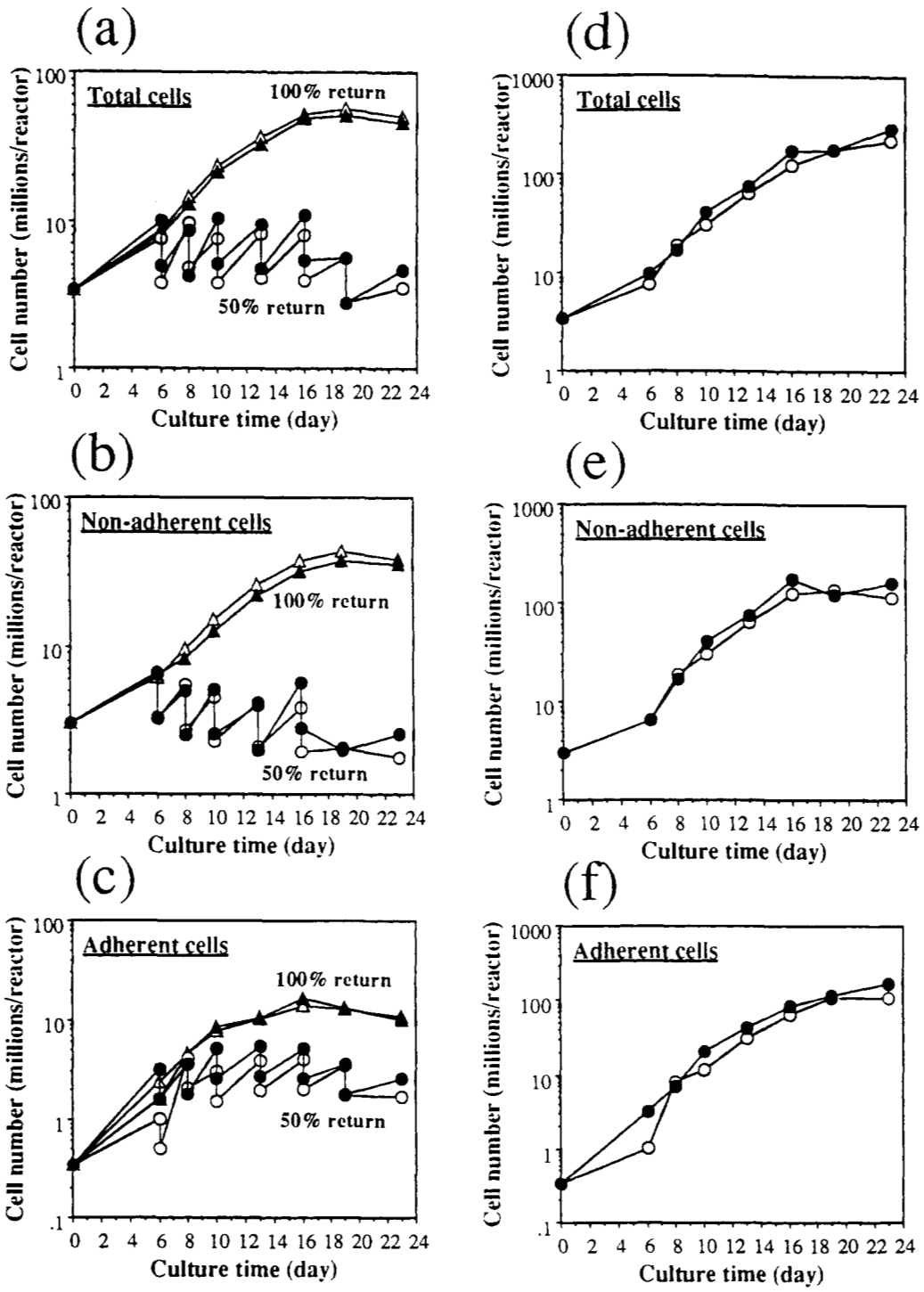

Figure 2. Time courses of total, adherent, and nonadherent cell numbers in perfused T-flasks repeatedly harvested and the corresponding calculated total cell production. Cells (3.4 million) were inoculated into each perfused T-flask (25 cm^2) and cell harvesting was initiated on day 6. In three untouched controls total, nonadherent, and adherent cell counts at day 15 were $8.34 \pm 0.64 \times 10^7$, $6.40 \pm 0.77 \times 10^7$, and $1.93 \pm 0.18 \times 10^7$, respectively. Panels (a), (b), and (c): primary data on cell numbers harvested for 50% and 100% cell return. Panels (d), (e), and (f): total cell numbers calculated based on the cell count at 50% cell return. These calculations are based on the assumption that the two identical cell populations would have grown the same way following replating. Only one cohort was actually cultured. In panel (d) the final total cell number is 260×10^6 per flask, and therefore, the overall expansion is 76.5.

$14^{6,7}$ has the potential to continue growing once the nonadherent cells are removed. Removing a fraction of the nonadherent cells at day 14 showed that cell growth continued following cell sampling (Fig. 1). Further, greater fractional removal of cells on day 14 yielded better return of total cell numbers.

These two experiments demonstrate that the cell population initially seeded has the potential to expand to greater

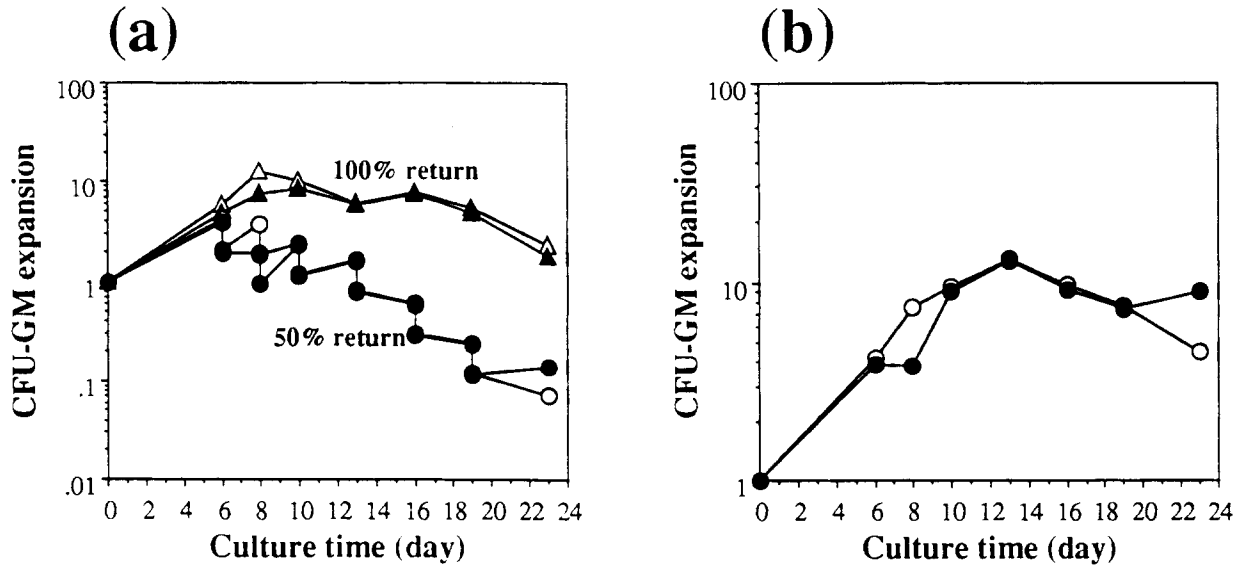

Figure 3. Time courses of CFU-GM expansion in perfused T-flasks repeatedly harvested. Initial CFU-GM density was 71.5 CFU-GM/20,000 total cells. Culture conditions were described in Figure 2. In 3 untouched controls, CFU-GM expansion at day 15 was 17.74 ± 1.93. (a) CFU-GM expansion; (b) the total CFU-GM expansion calculated based on the cell count at 50% cell return.

numbers than allowed by a single batch expansion, without losing the CFU-GMs. The next step was to carry out cultures in which cells were repeatedly harvested over time to increase the overall expansion attainable from a single aspirate.

Based on the results presented above, we began the sampling procedure at relatively low cell densities and harvested both the adherent and the nonadherent cell populations. The latter feature would allow the adherent cell layer to continue to grow and not reach confluence. Further, this cell harvesting procedure results in two identical populations; one that is harvested and discarded, and one that continues growing. Thus, the cells removed can, in principle, be inoculated into an identical perfusion culture.

The net result of this procedure increased the surface area for growth over time. Implementing this protocol in the parallel plate perfusion chambers described in ref. 7 is cumbersome, requiring repeated cell harvests. Thus, these experiments were carried out in perfused T-flasks where harvesting was more easily carried out. Perfused T-flasks, however, do not perform as well as perfusion bioreactors due to difficulty in volume and flow rate control (see below) but they allow easy implementation of the frequent harvesting protocol. The cell expansion ratios obtained in these experiments are thus conservative estimates for those that can be obtained in perfusion bioreactors that offer homogeneous and well-defined steady cellular environments.

The results from repeated harvesting experiments show that an overall cell expansion of over 70-fold was obtained by frequent cell harvesting and replating after day 6 of culture (Fig. 2). However, the total number of calculated progenitor cells produced did not significantly differ from the control cultures with 100% return of total cells harvested (Fig. 3). Further, the number of CFU-GM progenitor cells peaked around day 14 in the frequently harvested cultures. We have reported earlier that the CFU-GM numbers peaked around day 14 in untouched reactors.[6,7] The cultures in which all cells were removed and returned every 2 to 4 days showed an early maximum in the expansion of CFU-GM on days 8 to 10. This early peak and low CFU-GM expansion may be due to the frequent cell handling procedures probably affecting the microcellular structure established already. Alternatively, the early time (day 6) at which the harvesting procedure was initiated was disruptive. Further, removing 50% of the cells every 2 to 4 days led to a decrease in nonadherent cells and an overgrowth of the adherent cell layer (Fig. 2). Thus, the cultures display imbalance with respect to the relative growth of the nonadherent cells and the adherent cell population. This imbalance could possibly be corrected by uneven passing ratios of the adherent and the nonadherent cells.

A second series of repeated harvesting experiments was performed to address both shortcomings of the first set. The harvesting protocol was modified in three ways. First, the partial harvesting procedure was initiated at day 11. Second, the harvesting frequency was reduced to minimize the negative effects of the disruption of the cell bed on cell growth. Third, the adherent layer was also passed at a different ratio than the nonadherent cells. Further, because the stem cells are believed to be contained in the adherent layer, we further fractionated this population with a 1-h adhesion procedure (see Materials and Methods for details). This procedure separates most of the hematopoietic cells contained in the adherent cell population from the adherent stromal cells (mostly fibroblasts). The hematopoietic cells so obtained were pooled with the nonadherent cell populations.

This modified frequent harvesting protocol showed two important results. First, the calculated total cell population was expanded at least 100-fold (Fig. 4). This expansion was

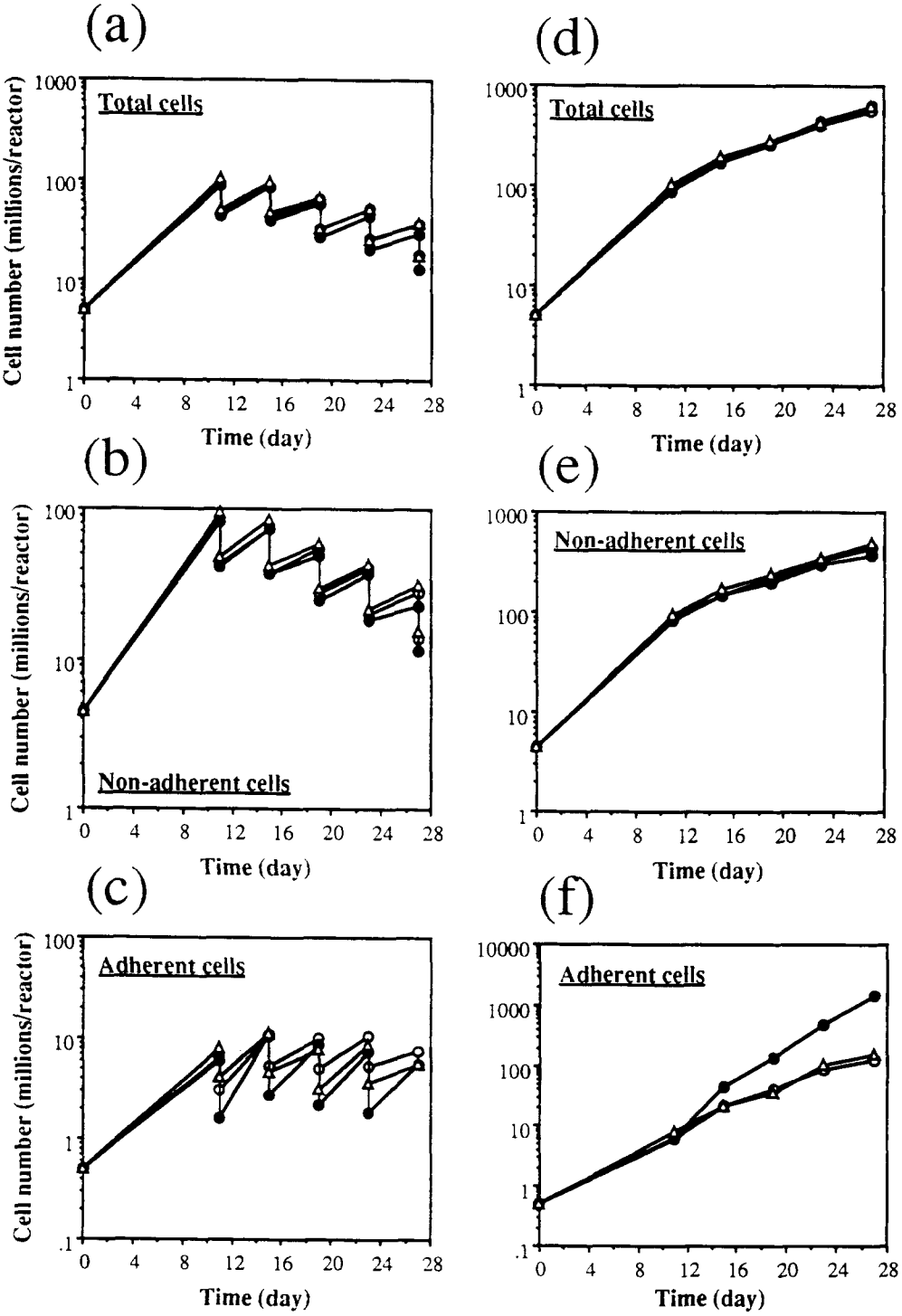

Figure 4. Time courses of the cell numbers in perfused T-flasks repeatedly harvested and the total cell production calculated. Five million cells were inoculated into each perfused T-flask and cell harvesting was initiated on day 11. In 2 untouched controls, total, nonadherent, and adherent cell counts at day 15 were $1.29 \pm 0.17 \times 10^8$, $1.19 \pm 0.16 \times 10^8$, and $1.04 \pm 0.07 \times 10^7$, respectively. (○): 50% total cell return; (●): 50% nonadherent and 25% adherent cell return; (△) 50% nonadherent, 50% nonstromal, and 10% stromal cell return from adherent cell population. Panels (a), (b), and (c): primary cell counts. Panels (d), (e), and (f): calculated total cell numbers based on the assumption that the harvested cells were replated and grew the same way as the fraction that was actually cultured. In panel (d), the final total cell number is 601.6×10^6 per flask, and therefore, the overall expansion is 120.

obtained over a time period of 27 days. Second, a sustained production of CFU-GMs was obtained (Fig. 5). In the case where 25% of the adherent cells were returned, total cell and CFU-GM expansion were the same as in the control. This protocol showed higher expansion of the adherent cell population. The replating protocol, where the hematopoietic cells that were contained in the adherent cells were separated from the stroma and pooled with the nonadherent

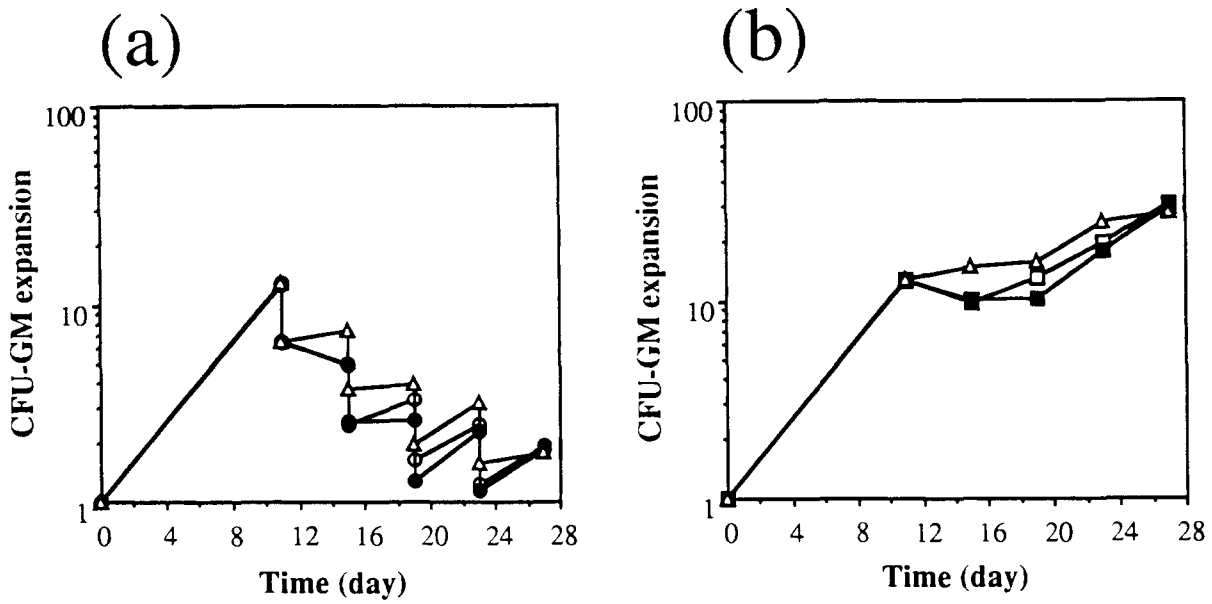

Figure 5. Time courses of CFU-GM expansion in perfused T-flasks repeatedly harvested and the calculated cumulative CFU-GM production. Culture conditions were described in Figure 4. Initial CFU-GM density was 129 CFU-GM/20,000 total cells. In the 2 untouched controls, CFU-GM expansion at day 15 was 9.56 ± 0.60. (○): 50% total cell return; (●): 50% nonadherent and 25% adherent cell return; (△): 50% nonadherent, 50% nonstromal, and 10% stromal cell return from adherent cell population. (a) CFU-GM expansion, counted; (b): total CFU-GM expansion, calculated based on the assumption that the harvested cells were replated and grew the same way as the fraction that was actually cultured.

fraction, resulted in almost the same expansion of total and progenitor cells as the other two replating protocols. Finally, the production of nonadherent cells does diminish with time (Fig. 4b).

DISCUSSION AND CONCLUSIONS

Obtaining sustained and balanced human hematopoiesis ex vivo is a major tissue engineering challenge with significant scientific and clinical implications. Prior to the present study, the recent literature showed that the medium exchange rate was a key variable in ex vivo human bone marrow cultivation[8,9] and that single batch expansion of MNCs leads to a 10- to 30-fold expansion of the various cell compartments.[6,7] Assuming that the current stem cell model of hematopoiesis (e.g., see ref. 2) is correct, then, under totally simulated in vivo conditions, sustained expansion of all hematopoietic cell compartments should be attainable ex vivo.

Single batch expansion leads to cell densities of about 3 to 4 × 10^6 cells/cm^2.[6,7] At these cell densities, the cell layer is on the order of five to ten cell layers in thickness. Given the fact that in vivo almost no cells are this far from a nutrient and oxygen source, one may expect that these high cell densities represent saturation of the cell carrying capacity of the surface. This "crowding" hypothesis was the basis for the work presented herein.

Previous bioreactor data suggest that the inoculated cells lead to the commitment of a number of early cells that reach the progenitor stage at about days 10 to 17.[7] Then, probably due to space limitations, further differentiation and production of postprogenitor stage cells does not occur. However, we know that the number of LTC-ICs increases over time.[6] The performance of the single pass expansion protocol, however, does enable clinical application to bone marrow transplantation.

The present data show that in the frequently harvested perfusion cultures, where the space available for growth is expanded, an extended growth period and expansion in cell number may be obtained. This increase in space led to a much higher production of mononuclear cells that were at the precursor and more mature stages of differentiation than obtained under the single batch expansion protocol. Furthermore, continued expansion of progenitor cells was obtained. The increased surface area with time leads to effectively lower cell surface densities and thus changes the rate of mass transfer of oxygen, nutrients, and protein to the cell bed. Limitations in any of these factors are alleviated. The extended growth data was obtained in perfused T-flasks because of the easy cell harvesting and replating features of the T-flask. However, these perfused T-flasks still suffered from several shortcomings. Liquid level control was difficult and fluid flow distribution was not good. Necrotic cell beds were observed in poorly perfused regions. Further, the enrichment of progenitor cells was not observed during the first 10 to 14 days of culture, as was reported to occur in bioreactors under similar conditions.[7] Thus, in a well-controlled bioreactor environment, the performance of the cell sampling protocol should be improved over the performance reported here.

The overall conclusion from the present study is that expansive human hematopoiesis ex vivo can be obtained in continuous perfusion culture for longer periods than previ-

ously reported. The extended expansion is consistent with the expansion of LTC-ICs during a single batch expansion culture.[6] The presence of an increased number of LTC-ICs after 14 days of culture suggests that further growth should have been possible. Thus, the previously published LTC-IC data and the extended growth data reported here are consistent.

The results presented are not final. Further optimization of this culture protocol is likely to lead to even longer growth periods and greater cell expansion. Such optimization needs to be focused on other factors in addition to the provision of adequate surface area for cell growth over time. Hopefully, optimization of culture conditions will lead to prolonged growth periods that can be carried out for arbitrary lengths of time.

Although useful for scientific and assay purposes, the frequent cell replating procedure is most likely unattractive in a clinical setting where minimal manipulation of cells is desired. It would be more desirable to simply increase the cell growth area at a few discrete points in time and harvest the entire cell population at the end of the culture. Clearly, this represents a major challenge for the next generation of designs for hematopoietic bioreactors.

We thank Drs. Stephen G. Emerson and Michael F. Clarke of the University of Michigan Medical Center for providing bone marrow samples. This work was supported by a postdoctoral fellowship to Dr. Duk Jae Oh, from Aastrom Biosciences, Inc., Ann Arbor, MI.

References

1. Eaves, C. J., Cashman, J. D., Eaves, A. C. 1991. Methodology of long-term culture of human hematopoietic cells. J. Tissue Culture. Meth. **13**: 55–62.
2. Emerson, S. G. 1991. The stem cell model of hematopoiesis, pp. 72–81. In: R. Hoffman et al. (eds.), Hematology: basic principles and practice. Churchill Livingstone, New York.
3. Greenberger, J. S. 1984. Long-term bone marrow culture: Proceedings of a symposium at the Kroc Foundation, Santa Ynez Valley, CA, Sept. 12–16, 1983, pp. 203–242.
4. Koller, M. R., Palsson, B. O. 1993. Tissue engineering: Reconstitution of human hematopoiesis ex vivo. Biotechnol. Bioeng. **42**: 909–930.
5. Koller, M. R., Bender, J. G., Miller, M. W., Papoutsakis, E. T. 1993. Expansion of primitive human hematopoietic progenitors in a perfusion bioreactor system with IL-3, IL-6, and stem cell factor. Bio/technology **11**: 358–363.
6. Koller, M. R., Emerson, S. G., Palsson, B. O. 1993. Large-scale expansion of human hematopoietic stem and progenitor cells from bone marrow mononuclear cells in continuous perfusion cultures. Blood **82**: 378–384.
7. Palsson, B. O., Paek, S. H., Schwartz, R. M., Palsson, M., Lee, G. M., Emerson, S. G. 1993. Expansion of human bone marrow progenitor cells in a high cell density continuous perfusion system. Bio/technology **11**: 368–372.
8. Schwartz, R. M., Palsson, B. O., Emerson, S. G. 1991. Rapid medium perfusion rate significantly increases the productivity and longevity of human bone marrow cultures. Proc. Natl. Acad. Sci. **88**: 6760–6764.
9. Schwartz, R. M., Emerson, S. G., Clarke, M. F., Palsson, B. O. 1991. *In vitro* myelopoiesis stimulated by rapid medium exchange and supplementation with hematopoietic growth factors. Blood **78**: 3155–3161.