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INTERFERON BETA 2/INTERLEUKIN-6 AND INTERLEUKIN-3 SYNERGIZE IN STIMULATING PROLIFERATION OF HUMAN EARLY HEMATOPOIETIC PROGENITOR CELLS.

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Abstract

Early 4-hydroxyperoxycyclophosphamide (4-HC) resistant hematopoietic progenitor cells (pre-colony-forming units, pre-CFU) were evaluated by a two-step liquid culture system, (earlier progenitors), pre-CFU, as well as by the conventional semi-solid mixed colony assay (later progenitors) for their growth response to interleukin-6 (IL-6), interleukin-3 (IL-3), and a combination of both factors. The effect of the IL-6/IL-3 combination was compared to that of IL-1/IL-3. IL-3 alone proved less effective in supporting earlier pre-CFU cells than later progenitor cells. In a previous work IL-6 promoted the growth of early multipotential progenitor cells circulating in hairy cell leukemia (HCL) patients. IL-6 alone did not stimulate growth of either early or later normal progenitor cells. However, a significant synergistic effect was obtained when IL-6 and IL-3 were added together ($p<0.05$). IL-6/IL-3 synergism was more potent than IL-1/IL-3 in promoting growth of colonies. The previously described synergistic effect of IL-1/IL-3 seems to be independent of IL-6. Thus, our results suggest that the multifunctional cytokine IL-6, may be of use in shortening the engraftment time in bone marrow transplantation.

KEY WORDS: IL-6/IFN-β2; IL-3; Colony forming cells; Colony assays; Stem cells; Colony stimulating factors; Granulocytic/Macrophage colony forming units; Burst forming units Erythroid; Multipotential progenitors; Interleukins.

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Introduction

The formation of mature blood cells comes at the end of a process which starts by the proliferation of specifically committed progenitor cells called colony forming units (CFUs). Progenitor cells as well as mature blood cells are transit populations with little capacity of self-generation (2,11). Progenitor cell numbers are maintained by the proliferation of a pre-CFU population among them are early hematopoietic stem cells. These cells are known to reside in a non-cycling stage Go (8) but after entering the cell cycle they possess a distinct self-generation capacity.

Early human stem cells are difficult to isolate and identify. Early progenitor cell populations can be enriched by positive selection with the CD34 (My10) monoclonal antibody (3), or by negative selection of all known accessory cells and committed progenitors. Some investigators use a blast colony assay to assess the early pre-CFU population (14). 4-hydroperoxycyclophosphamide (4-HC) has a sparing effect on non-cycling early stem cells (5), and eliminates committed progenitors (13). This is supported by the observation that 4-HC treated marrow is capable of reconstituting normal hematopoiesis following autologous bone marrow transplantation (21). 4-HC and further enrichment by a two-step system, in which pre-CFU cells are "primed" in liquid culture for one week, was used in our laboratory to study the effects of different colony stimulating factors (CSFs) on the pre-CFU cells.

Interleukin 3 (IL-3) has been shown by many investigators to be a multipotential CSF (4,15) acting on multipotential progenitors, i.e., CFU-Mix or CFU-GEMM (colony forming unit granulocyte, erythroid, macrophage and megakaryocyte), but has a lesser effect on pre-CFUs. Other CSFs such as GM-CSF (granulocyte/macrophage CSF) and M-CSF (macrophage CSF) act on committed progenitors.

Hematopoietin-1 (HP-1), previously termed synergistic factor, induces stem cells to become responsive to other CSFs (12). HP-1 acts synergistically with IL-3 in supporting proliferation of early hematopoietic progenitors (1,25). It is now evident that most of the HP-1 activity is due to Interleukin-1 alpha (IL-1) (17). Recently a

combination of Interleukin-6 also named Interferon-ß-2 and IL-3 proved superior to IL-1 and IL-3 in supporting colony formation in 2-day post 5FU murine bone marrow (BM) cells (9).

Interleukin 6 also named Interferon Beta 2 (IL-6/IFN-ß2) was first described and cloned from dsRNA induced human fibroblasts by its beta IFN type anti-viral and anti-growth activity (22, 27). IFN-ß2/IL-6 was found to be identical with B-cell stimulating factor (BSF-2) and also with hepatocyte stimulating factor (HSF) (20). This multifunctional cytokine is produced in a regulated fashion by a variety of normal cells (e.g., fibroblasts, endothelial cells and monocytes). The name IL-6 will be used in this work.

IL-1, tumor necrosis factor (TNF), platelet derived growth factor (PDGF) and other cytokines trigger expression of IL-6 in fibroblasts (23). Thus, the possibility of an indirect hematopoietic effect of IL-1 via IL-6 on stem cells was also evaluated in the present work.

Earlier studies in our laboratory showed that IL-6 was active on a special progenitor cell with multipotential capability: lympho-GEMM (LGEMM), found in peripheral blood (PB) from patients with Hairy Cell Leukemia (HCL) (16,20). These cells may represent earlier cells than the CFU-Mix. The present work further demonstrates that IL-6 also acts on normal early hematopoietic progenitors.

Materials and Methods

Bone Marrow Samples

Normal human bone marrow (BM) was obtained with informed consent (according to the guidelines of the Helsinki Committee) from adults in whom the bone marrow was not involved with any pathology.

Peripheral Blood Samples

PB samples were obtained from healthy adult laboratory workers and also from HCL patients.

Cell Separation Procedure

BM and PB aspirated into preservative free heparin were separated over a Ficoll-Hypaque gradient (Pharmacia) at 400g for 30 min and the interface mononuclear cells collected, washed 3 times and resuspended in Iscove's Modified Dulbecco's Medium (IMDM). T-cell depletion was performed using AET treated sheep red blood cells followed by a second centrifugation on a Ficoll-Hypaque gradient. The T-depleted fraction was then depleted of macrophages by adherence to plastic at 37^0C overnight in IMDM with 15% fetal calf serum (FCS). Preparation purity was over 90%.

4-hydroperoxycyclophosphamide (4-HC) treatment

To enrich for early human progenitors, low density non-adherent, non-rosetting BM and PB cells were treated with 4-HC. Briefly, the mononuclear cells were incubated with 4-HC at 100µg/ml (as determined by a dose response curve) for 45 min at 37^0C with frequent agitations and washed three times with cold IMDM.

Colony Assays

Day-0 cultures: 10^5 cells were plated in 1ml of IMDM containing 0.9% methylcellulose, $5x10^5$ mg/ml 2-mercaptoethanol, 10% FCS, 20% fresh frozen plasma and when indicated 7.5% vol/vol PHA-LCM (phytohemagglutinin leukocyte conditioned medium). All plates contained 1 U/ml recombinant erythropoietin (Connaught, Ontario, Canada). The cultures were incubated in a fully humidified atmosphere containing 5% CO_2 at 37^0C for 14-16 days. When recombinant growth factors were added to the cultures no PHA-LCM was included.

Liquid Culture: The cells used in this two-step system (10) were treated as above with 4-HC, resuspended at 10^5 cells/ml in IMDM containing 10% FCS, different growth factors and incubated for 5 to 7 days. Control cultures with PHA-LCM as a pool of growth factors were included. At the end of the incubation period, the cells were pelleted and plated in the methylcellulose assay as in day-0 cultures, with PHA-LCM as a source of CSFs. The increase in the number of secondary colonies reflects the expansion of early progenitors in response to the primary stimulus during the liquid phase.

Definition of Colony Types

Colonies were classified according to their appearance, using an inverted microscope, (Nikon, Diaphot) as: CFU-Mix (colony forming units granulocyte, erythroid, macrophage and megakaryocytic) CFU-GM (colony forming units granulocyte/macrophage), BFU-E (burst forming units erythroid). The identity of cells within colonies is routinely checked by picking out colonies, spreading on slides and staining with Giemsa.

Hematopoietic Factors

Recombinant IL-6 (rIL-6) was supplied by M. Revel and M. Rubinstein (Interpharm Nes-Ziona, Israel). rIL-6 from mammalian cells was produced in Chinese Hamster ovary cells (CHO) clones (28). Bacterial rIL-6 was produced in E.coli (7). rIL-6 was used at concentrations 0.3 to 30 U/ml.

Recombinant Interleukin-3 (rIL-3) was used at 10 U/ml.

Recombinant Interleukin-1 (rIL-1) was used at 2 U/ml.

Monoclonal anti-IL-6 antibodies from hybridoma clone 34 were supplied from A. Zilberstein (Weizmann Inst.). Dilutions of 1:100 to 1:500 in IMDM were used in the experiments.

Statistical Analysis

Mean and Standard error of mean (SEM) for colonies grown in duplicate dishes were assessed and sets of data compared using the Wilcoxon's Rank test for unpaired samples.

Results

Effect of rIL-6 on hematopoietic colony formation in HCL-PB:

During our studies on the growth of hematopoietic colonies from peripheral blood (PB) of HCL leukemia patients (16), we observed that the addition of IFN-alpha or beta-1 species produced a slight but consistent stimulation in contrast with the marked inhibition seen on normal hematopoietic progenitors. The addition of rIL-6 (CHO) to PHA-LCM when compared to IFN-alpha, produced a much larger stimulation of colony growth in HCL-PB (not shown). rIL-6 alone also stimulated growth of progenitor cells in HCL-PB as shown in fig. 1. A dose response curve of colony formation in HCL PB was performed. The

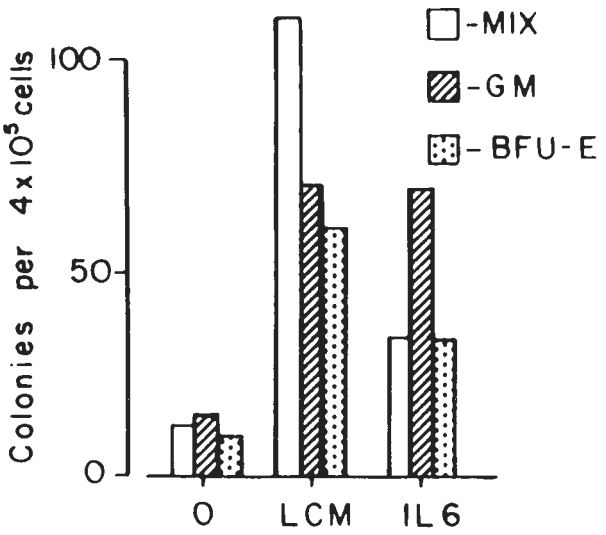

Figure 1. Effect of rIL-6 (CHO) 0.3 U/ml on PB progenitor cells from HCL patients in liquid cultures. Results are mean of 6 independent experiments in duplicates. (SD less than 10%).

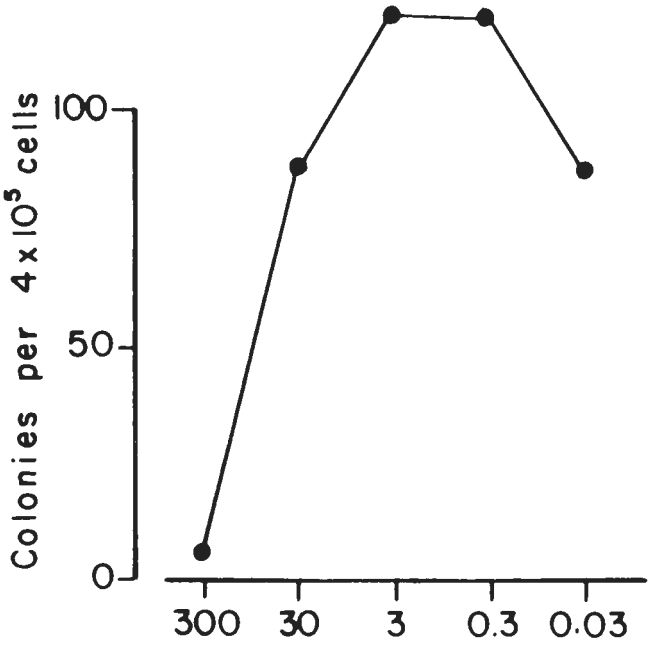

Figure 2. Dose response curve of rIL-6 (CHO) on HCL progenitors. Above 30 U/ml, rIL-6 shows cytoxicity.

optimal concentrations were 0.03 to 30 U/ml of recombinant IL-6 (fig.2). In our experiments we used 0.3 U/ml (anti-viral U/ml).

Effect of rIL-6 on normal BM hematopoietic colony formation

Monocyte and T-depleted 4-HC treated normal bone marrow cells grown in the day-0 culture assay with addition of rIL-6 (CHO), showed no significant increase in CFU-GM, BFU-E and CFU-Mix. These results greatly differed from the 5 fold stimulation in colony growth produced by rIL-3 alone (fig.3). rIL-6 (CHO) when added to rIL-3 however, promoted a slight increase as compared to rIL-3 alone (20% increase of all colony types). These cultures were grown without PHA-LCM.

We next compared the combination rIL-6 (CHO) and rIL-3 on earlier progenitor cells in the liquid two step assay. rIL-3 alone was much less effective than in the day-0 cultures. The increase in colony formation with rIL-3 was only slightly greater than rIL-6 alone (fig.3). rIL-3 in day-0 cultures replaced PHA-LCM completely, but had a much less prominent effect on the earlier quiescent pre-CFU cells. When the pre-CFU cells were incubated with rIL-3 and rIL-6 (CHO), a clear synergism was demonstrated (fig. 3). When analyzing different progenitor cell types, a major increase in CFU-GM was evident in day-0 cultures while in the liquid assay an increase occurred mainly in BFU-E and CFU-Mix (fig.4). It is possible that the cycling progenitors namely CFU-GM were no longer available in the liquid assay and therefore rIL-6/rIL-3 could not influence their proliferation.

rIL-6 produced in E.coli induced similar effects to CHO (fig.5). When added alone, no stimulation of colony growth was obtained in day-0 cultures (left columns, fig.5) while a slight stimulation of colony growth was observed in the

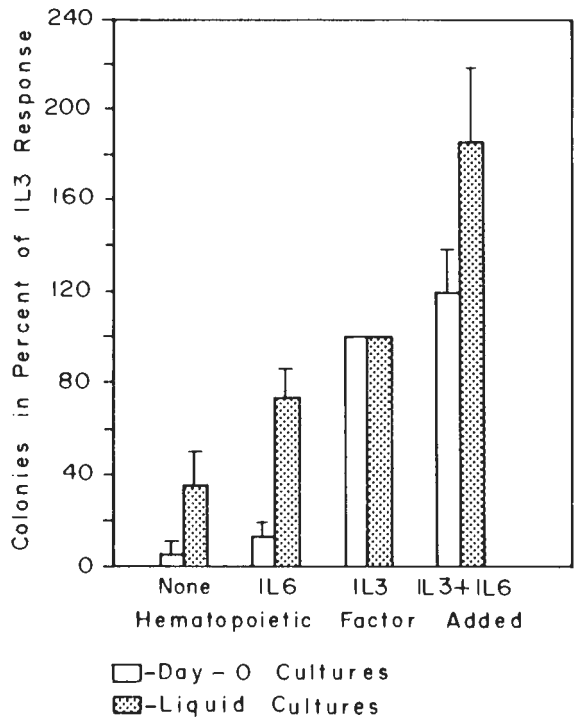

Figure 3. Comparison between day-0 (left columns columns) and liquid cultures (right columns) using rIL-6 (CHO) 0.3 U/ml. 10^5 depleted 4-HC treated cells per ml of culture plated. Results are mean ±SEM of 5 experiments in duplicates and are expressed as increase percent in relation to IL-3 response, considered as 100%.

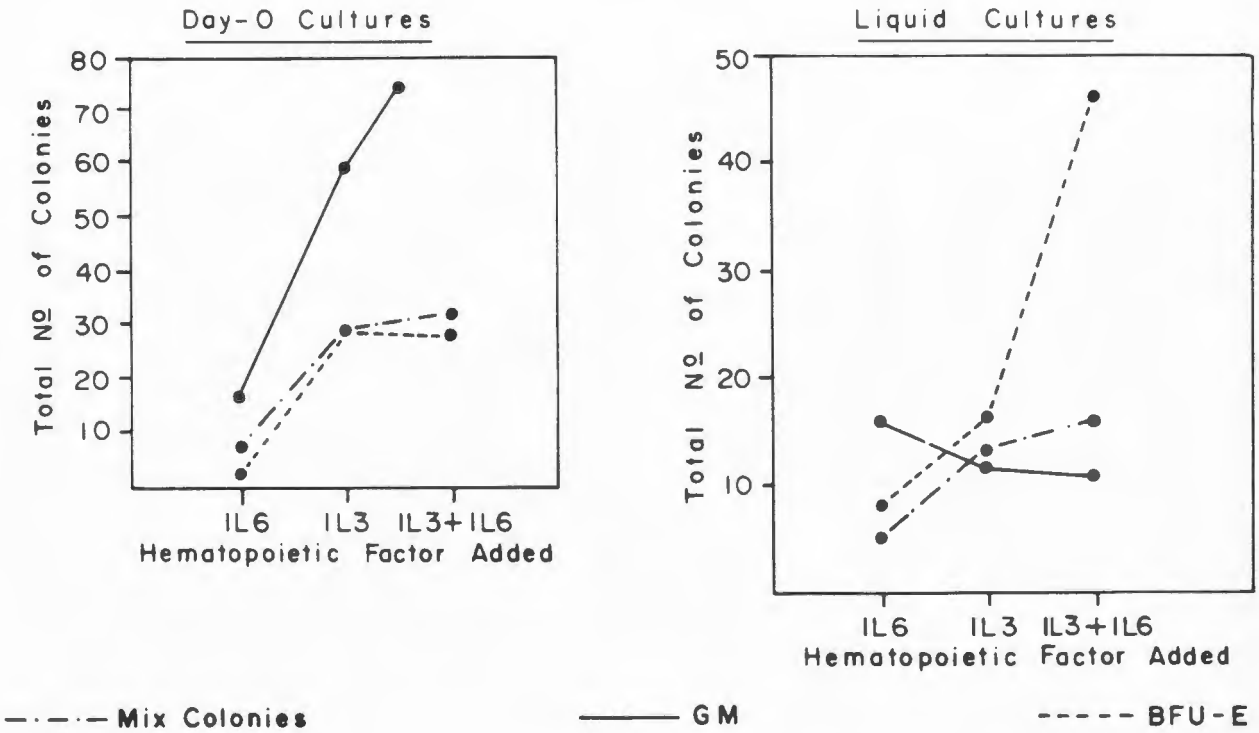

Figure 4. Different colonies (CFU-GM, BFU-E and CFU-Mix) obtained with rIL-6 (CHO), rIL-3 and rIL-6/rIL-3 combination, evaluated in day-0 cultures and liquid cultures. 10^5 depleted 4-HC treated BM cells per ml of culture. Results are mean of 5 experiments in duplicates. rIL-6 (CHO) 0.3 U/ml; rIL-3, 10 U/ml. Day-0 and liquid cultures are shown on right and left pannel, respectively.

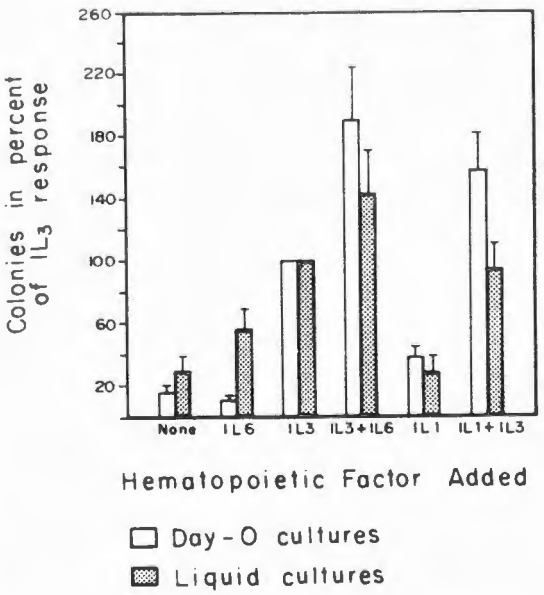

Figure 5. Comparison between day-0 and liquid cultures using rIL-6 (E.Coli) 3 U/ml. Results are mean ±SEM of 7 experiments in duplicates and are expressed as increase percent in relation to IL-3 response considered 100%. Significant synergism between rIL-6/rIL-3 as compared to rIL-3 ($p<0.05$).

liquid cultures (right columns, fig. 5). A significant synergism was obtained when both rIL-3 and rIL-6 were added together in the two assays as shown in fig. 5 ($p<0.05$). The number of all colonies increased in day-0 cultures with the combination of rIL-6 and rIL-3 (fig.6). In the liquid cultures a pronounced increase was seen in CFU-Mix, a small effect in BFU-E and almost none in CFU-GM (fig.6), i.e., rIL-3 alone promoted growth of 15 CFU-Mix colonies as compared to 35 CFU-Mix with rIL-6/rIL-3 (fig.6). The results shown in figures 3 and 5 relate to the number of colonies obtained with rIL-3 and are expressed as 100%. The calculations of percent increase are derived from the number of colonies scored, shown in figs. 4 and 6, respectively.

rIL-1 when added alone to the cultures behaved similarly to rIL-6 and did not promote growth of colonies. However, synergism between rIL-1 and rIL-3 was observed (fig. 5). In experiments comparing the combination rIL-1/rIL-3 versus rIL-6/rIL-3, the former combination proved less effective in stimulating growth (fig.5). To check whether rIL-1/rIL-3 synergism is due to production of IL-6 by some remaining accessory cells we used anti-IL-6 monoclonal antibodies. The addition of anti-IL-6 antibodies did not alter the synergism and no additive effects were obtained with rIL-1 and rIL-6, suggesting that the two cytokines act each on its own (data not shown).

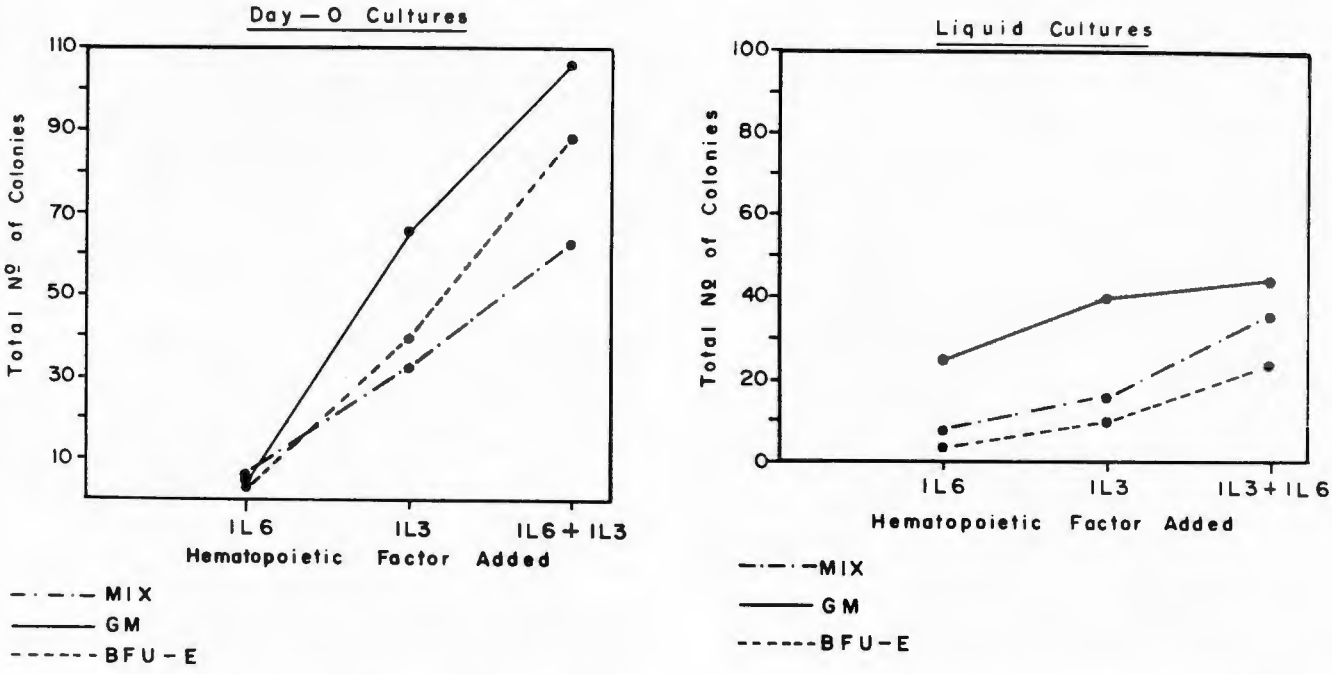

Figure 6. Different colonies (CFU-GM, BFU-E and CFU-Mix) obtained with rIL-6 (E.Coli), rIL-3 and a combination of both in day-0 cultures (left) and liquid cultures (right). 10^5 depleted 4-HC treated BM cells per ml of culture. Results are mean of 7 experiments in duplicates. rIL-3, 10 U/ml rIL-6, 3 U/ml.

Discussion

The results of our study show that rIL-6 plays a role in "priming" early progenitor cells to respond to rIL-3. To study pre-CFU cell responses, T and monocyte depleted bone marrow cells after 4-HC treatment were "primed" in the liquid two step culture assay with hematopoietic factors including rIL-6, and then checked for growth response in methylcellulose assays. Using this model, we show that rIL-6 by itself does not support colony formation indicating that it does not act by itself as a growth promoting CSF. Only when both rIL-6 and rIL-3 are present a clear synergism can be demonstrated. Different types of progenitor cells were stimulated to grow by the rIL-6 and rIL-3 combination. In the two step system, using either CHO or E.Coli rIL-6, mainly BFU-E and CFU-Mix colonies proliferated while in the day-0 cultures CFU-GM predominated (fig.6). This variability in cell type response has not been described previously, and may suggest two different hematopoietic effects of rIL-6: A priming effect on pre-CFU cells and a differentiating effect on granulocyte/macrophage lineage. rIL-6 has also been shown to be capable of inducing differentiation of leukemic mouse cells (24) and increase of receptors for IL-3 and GM-CSF has been reported for IL-6/IFN-β2/MG12 on leukemic cells (19,24). The lack of effect on CFU-GM may also be due to the fact that these are later progenitors and maybe eliminated by 4-HC in the 5 days cultures, therefore not responding to rIL-6.

Interleukin 1 is another cytokine capable of priming pre-CFU cells to respond to rIL-3 (18). We have confirmed the rIL-1/rIL-3 synergism and found that rIL-6/rIL-3 is a more active combination. Both rIL-1 and rIL-6 are multifunctional cytokines involved in the immune response and other important biological processes. Induction of IL-1 by IL-6 accounting for the pre-CFU cells response seems improbable since addition of monoclonal antibodies to IL-6 did not abolish the IL-1/IL-3 effect and no additive growth responses were obtained when both rIL-1 and rIL-6 were used together.

It is not yet clear whether IL-6 acts directly on the stem cells. To answer this question, higher purity of target cells i.e., CD34 My10 positive cells and the 4-HC two step assay are needed. Possible influence of T accessory cells known to be activated by IL-6 (26), or influence of other factors produced by stromal elements in the microenvironment also remain to be evaluated. In any case, the synergistic effect obtained with rIL-6 and rIL-3 suggests that this combination may be useful in fastening reconstitution of hematopoiesis in bone marrow transplantation. The IL-6/IL-3 synergism may prove equal or superior to other combinations already tried in vivo (6).

Acknowledgements

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Discussion with Reviewers

H. Gamliel: The present study used light microscopy and cytochemical stains to identify colonies. Indeed, it appears that light microscopy might suffice to distinguish between Mix, GM and BFU-E colonies. Would you agree that electron microscopy in conjunction with immunolabeling may enable even better identification of colonies and sub-colonies, e.g., G and M subcolonies etc.? Also, why lymphocytes are not represented in any of the above colonies? Should not they be part of the CFU-Mix colonies?
Authors: Electron microscopy with immunolabeling would be of great help in identifying lymphocytes. It may be too cumbersome for a routine work or big number of experiments but in the context of a pertinent research it would definitively add in characterization of lymphocytes. There are no lymphocytes (by morphology) that grow in mixed colonies (CFU-Mix) from normal blood or bone marrow and the reason for it is not yet entirely clear. Some attribute it to the age of progenitor cells that are allowed to grow under the given culture conditions but it may also be that some factors are lacking that promote lymphocytic differentiation. Lymphocytes derived from a common lymphomyeloid progenitor have been described in stem cell diseases and, recently by us, in hairy cell leukemia (ref 16).

H. Gamliel: Could you comment on what actually triggers early hematopoietic stem cells to enter the cell cycle, mainly, if priming pre-CFU cells, as presently reported, also leads to changes in the self generation capacity of these cells, besides turning the cells responsive to other growth factors?
Authors: Early hematopoietic stem cells are quiescent cells (Go phase) and there is lack of knowledge as to what triggers them to enter the cell cycle. It seems that IL-6 may be capable of doing this by promoting the cells to respond to IL-3. Other candidates would be G-CSF and IL-1. This question is crucial and I believe that the answer will be possible when true stem cells are isolated and fully characterized.

H. Gamliel: What are the actual numbers of pre-CFU cells that are left after treating a bone marrow aspirate? If for example you start with $10x10^6$ mononuclear cells, how many pre-CFU cells can be harvested from such a sample?
Authors: The liquid culture assay used for pre-CFU cells is a modification of the Johnson and Bradley type of assay for stem cells in the mouse. It is based on the concept of a quiescent state of stem cells and should allow enrichment for earlier non-cycling cells. The numbers of pre-CFU cells that are left after treating a bone marrow aspirate when exposed to IL-6 IL-3 are of a magnitude of approximately 1:1000. This can be augmented by thoroughly depleted samples with a pannel of monoclonal antibodies specific for lineage dependent cells and treatment with 4-HC or 5FU, that kill cyclin cycling cells and spare engrafting hematopoietic progenitor cells.

H. Gamliel: Could you briefly review reports on stem cells in the peripheral blood of leukemic patients, mainly, in hairy cell leukemia (HCL). Is it possible to positively identify such cells in blood samples from HCL just by immunolabeling them, e.g., for CD34? In cases of HCL, are the circulating progenitor cells different from the progenitor cells common to the B-cell and monocytic lineages?
Authors: The presence of lymphomyeloid stem cells with high self renewal and lymphoid differentiative capability in HCL was described by us in 1987 (16). We then showed that these primitive precursors bear the CD34 antigen (Leukemia Research, in press). Not all CD34 positive cells however are stem cells but rather consist of a frequency of 1:100, 1:50 at the best conditions. We do not know yet if these stem cells are normal stem cells circulating in the blood of HCL patients as a response to the fibrosis and leukemic process in the marrow, or if those are early cells that underwent a maturational arrest causing accumulation of preplasmatic B cells (hairy cells). A chromosomal or enzymatic marker would help clarify this important issue of cause or consequence. The cells common to B and monocytic lineages are not the same as the circulating progenitor cells in HCL. Little is known in man about characterization of the lymphoid-stem cell arm in hematopoietic development.

P.M. Grimley: How many different blood samples were examined to determine the statistical validity of IL-3/IL-6 synergism? While the Wilcoxon rank test is useful, data from just one sample would not necessarily indicate biologic consistency.
Authors: Five different marrow samples were examined to determine the IL-3/IL-6 synergism. This is stated in the legend for fig. 3.

P.M. Grimley: Have there been any cell cycle analysis of pre-CFU cells after one week in the liquid medium? Are such results consistent amongst samples from different individuals? Since marrow proliferative activity gradually declines with age, was this a factor with different donors? Do the colonies obtained from day-0 cultures and after priming differ statistically in cell cycle distributions, after for example IL-3 + IL-6 treatment shown in fig. 3?
Authors: We have not analysed cell cycle. It is however known that earlier stem cells are quiescent (in Go). In the Ogawa publication (text, ref. 15) cited in references there are some evidences suggesting that IL-6 may shorten the time in Go therefore enabling cells to respond to IL-3. It may be true that older donors may have less colony growth. We

have not examined thoroughly this question and thus cannot comment further on this point.

P.M. Grimley: I cannot find the data which supports your statement that IL-3 is much less effective in supporting pre-CFU cells assayed by the two-step liquid culture system. This is not apparent, for example in fig. 3.
Authors: You are entirely right. Our statement is not apparent if one looks only at fig. 3 where we choose to show the effects of rIL-6 and synergism between rIL-6 and rIL-3 as related to the rIL-3 activity. Therefore, the results of both day-0 and liquid cultures are shown as related to growth with rIL-3 (considered 100%). The differences are evident if one looks at the data showing actual colony numbers in figs. 4 and 6. IL-3 indeed was a less stronger inducer of colony growth in the pre-CFU assay as compared to its stimulatory effect on day-0 cultures on CFU-Mix. This is in accordance with the findings of most groups working on stem cells. IL-3 is a very potent stimulator of growth at relatively early stages of hematopoietic precursors but probably not on the earliest pre-CFU cells. The common adopted view of most workers in this field is that IL-3 is not the "stem cell factor" and its response is mainly on cells that were first primed and prepared to respond to IL-3 by earlier factors. Among these are IL-6 and IL-1 as well as G-CSF.