Secretion of Cytokines (Interleukins-1α, -3, and -6 and Granulocyte-Macrophage Colony-Stimulating Factor) by Normal Human Bone Marrow Megakaryocytes

By Claudia Wickenhauser, Johann Lorenzen, Juergen Thiele, Arne Hillienhof, Karsten Junghem, Beate Schmitz, Martin-Leo Hansmann, and Robert Fischer

The effects of cytokine stimulation [recombinant human interleukin (rHIL)-1α, rHIL-3, rHIL-6, rHIL-11, and rh granulocyte-macrophage colony-stimulating factor (GM-CSF)] on the secretory activity of normal human megakaryocytes were studied by means of the reverse hemolytic plaque assay (RHPA) in enriched cell preparations. This test facilitates an extremely sensitive determination of cytokine secretion at the single-cell level, together with the clear-cut identification of each immunostained (CD61+ secretory active megakaryocyte. Moreover, the reverse transcriptase-polymerase chain reaction (RT-PCR) was used to investigate the expression of IL-6, IL-6 receptor (IL-6R), IL-9, IL-10, IL-12, and IL-13 mRNA in highly concentrated megakaryocyte preparations. In comparison with the spontaneous secretion rate, stimulation with rHIL-1α, rHIL-6, and rhGM-CSF failed to induce a significant increase in the release of cytokines by CD61+ cells. On the other hand, both rHIL-3 and, in a less pronounced way, rHIL-11 exerted a marked effect on IL-6 secretion. Additionally, after stimulation with rHIL-3, a significant enhancement of the secretion of IL-3 and GM-CSF, but not of IL-1α, could be observed. Using the RT-PCR, a significant induction of IL-6 expression could be appreciated in the enriched megakaryocyte population (60% to 80%) stimulated with rHIL-3. The results of this study provide persuasive evidence that a number of cytokines are synthesized and secreted by human megakaryocytes and not only by hematopoietic stroma cells. These data suggest the existence of autocrine and paracrine mechanisms that may influence maturation and differentiation of megakaryocytes as well as act on various stroma cells to sustain an appropriate hematopoietic microenvironment.

© 1995 by The American Society of Hematology.

HUMAN MEGAKARYOPOIESIS is generally regarded as a complex phenomenon that includes proliferation of committed progenitor cells, cellular maturation with associated nuclear polyploidization (endoreduplication) and growth in size, and platelet production. These complicated processes are at least in part regulated by certain interleukins (ILs) and colony-stimulating factors (CSFs). Several recently published studies have provided strong evidence for the suggestion that a hypersensitivity or a reduced responsiveness to several cytokines may explain the proliferation of this cell lineage in myeloproliferative disorders (MPDs).

Additionally, pathologic release of certain cytokines probably takes part in the pathogenesis of the myelofibrosis, frequently accompanying MPDs. This hypothesis has especially stimulated a renewed interest in the study of megakaryopoiesis in both normal and disordered states of the bone marrow. However, the megakaryopoietic lineage represents only a small fraction of the nucleated cells within the human bone marrow. Hence, attempts to clarify the role of this peculiar cell type within the hematopoietic microenvironment meet certain technical difficulties. Failure to determine the secretory activity of megakaryocytes may be due to the fact that these mediators are acting at very low concentrations.

The present study was undertaken to assess cytokine release, i.e., the secretion of IL-1α, IL-3, IL-6, and granulocyte-macrophage (GM)-CSF by normal human megakaryocytes. For this purpose, we applied the reverse hemolytic plaque assay (RHPA) because of its high sensitivity and the possibility of identifying single actively secreting cell elements by immunochemical means. Further experiments using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique for the detection of IL-6, IL-6 receptor (IL-6R), IL-9, IL-10, IL-12, and IL-13 mRNA were performed using highly purified megakaryocyte preparations.

MATERIALS AND METHODS

Marrow cell preparation. Bone marrow specimens were obtained from the sternum of 50 patients undergoing thoracotomy for various surgical procedures. These patients presented without hematologic disorders and with platelet counts within the normal range. Informed consent was obtained from all donors. Marrow particles were immediately transferred to serum-free Iscove’s medium (GIBCO, Paisley, UK) containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 0.1% bovine serum albumin (BSA) purchased from Sigma (St. Louis, MO). Megakaryocytes were isolated from bone particles in 30-mm culture dishes (Greiner, Fridingenhausen, Germany) after gentle agitation for 4 hours at 37°C in a humidified atmosphere of 5% CO2 in air.

Megakaryocyte purification procedure. Megakaryocytes were enriched by density centrifugation at 400g over a single-density Percoll gradient (density, 1.050 g/mL; Seromed, Berlin, Germany) and washed twice in Iscove’s medium containing penicillin, streptomycin, and 0.1% BSA. The interphase was collected, and cells were resuspended to a final concentration of 2 × 106 to 3 × 106 cells per milliliter in RPMI 1640 supplemented with 0.1% BSA, penicillin (100 U/mL), and streptomycin (100 μg/mL). Before the RT-PCR procedure, Percoll interphase cells were first incubated with CD61 antibody (Y2/51, 10 μg/mL; Dako, Hamburg, Germany) for 10 minutes on ice and subsequently stained with anti-IgG1-MACS beads (1:5 dilution; Miltenyi Biotec, Bergisch-Gladbach, Germany) for 5 minutes at room temperature. Finally, the cells were separated over a miniMACS column (Miltenyi). A more detailed description of this method has been published previously.

RT-PCR. Megakaryocyte preparations and native bone marrow...
period of 8 hours. The megakaryocyte mRNA was isolated according to the instructions of the manufacturer (GIBCO-BRL, Eggenstein, Germany). After reverse transcription, 3 μL of the reaction mixture was diluted to 10^5 cells per milliliter in RPMI 1640 medium supplemented with 0.1% BSA and penicillin/streptomycin and incubated at 37°C in a humid atmosphere with 5% CO₂. Samples were stimulated by addition of 5 ng/mL recombinant human (rh) IL-3, or 10 ng/mL rhIL-6, and 10 ng/mL rhIL-11, in megakaryocyte medium.37 The MoAb was purchased from Dako.

**Table 1. Sequences of the Oligonucleotide Primers**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-GTGGGGGCCTTGGCCAGGACCA</td>
<td>548</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-ATGGACGGCCCTCCACAGAGA</td>
<td>628</td>
</tr>
<tr>
<td>IL-9</td>
<td>5'-GAGGGGCTCAGACGAGATGGC</td>
<td>295</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-AACGTTGAGGACAACACCAGC</td>
<td>305</td>
</tr>
<tr>
<td>IL-12</td>
<td>5'-CAAGACGGCATCATCACCATA</td>
<td>306</td>
</tr>
<tr>
<td>IL-13</td>
<td>5'-TGATTGGGACAGGGACACCCT</td>
<td>408</td>
</tr>
<tr>
<td>IL-6R</td>
<td>5'-CAAG CCTCCCAAGTGCAAGAT</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>3'-ATTGCTGATTGCTC ATAAGGGC</td>
<td></td>
</tr>
</tbody>
</table>

Sequences of the oligonucleotide primers used for RT-PCR to determine cytokine gene expression in the current study and the expected lengths of the amplification products are listed. Primer sequences for β-actin and IL-6 have been described previously.34 Primer design was facilitated by extensive European Molecular Biology Laboratory (EMBL) and GenBank database searches using the VAX-computer at the Department of Genetics, University of Cologne, as well as the Experimental GENINFO BLAST Network Service at the National Center for Biotechnology Information (NCBI), Bethesda, MD.34

fractions were diluted to 10^7 cells per milliliter in RPMI 1640 medium supplemented with 0.1% BSA and penicillin/streptomycin and incubated at 37°C in a humid atmosphere with 5% CO₂. Samples were stimulated by addition of 5 ng/mL recombinant human (rh) IL-1α, 5 ng/mL rhIL-3, or 5 ng/mL rhIL-6, respectively, over a period of 8 hours. The megakaryocyte mRNA was isolated according to a protocol described previously.34 Briefly, megakaryocytes were collected by centrifugation, and then the cell pellets were lysed in 200 μL of a solution containing 4 μmol/L guanidinium isothiocyanate, 25 mmol/L sodium citrate, 0.1 mol/L 2-mercaptoethanol, and 0.5% N-laurylsarcosine and were vortexed vigorously. After extraction with water-saturated phenol, chloroform, and chloroform:isoamyl alcohol (49:1 vol/vol), cellular mRNA was precipitated by adding 70% ice-cold ethanol and subsequently washed with isopropyl alcohol. The mRNA obtained was vacuum-dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water to a final volume of 20 μL. Reverse transcription was performed in a total volume of 20 μL using oligo (dT)₁₂ (Promega, SERVA Feinbiochemica, Heidelberg, Germany) as primer and the reverse transcriptase Superscript II, according to the instructions of the manufacturer (GIBCO-BRL, Eggenstein, Germany). After reverse transcription, 3 μL of the reaction solution was used for PCR amplification with the primers listed in Table 1. Using a hot start technique36 combined with a “touch down” temperature protocol,36 40 cycles were performed on a thermocycler (Perkin Elmer, Duesseldorf, Germany) using a sample volume of 50 μL. A second round of 40 PCR cycles was performed using 10 μL of the amplified material and fresh Taq-polymerase. The products were separated on 5% NuSieve agarose gels (Biozym Diagnostic, Hessisch Oldendorf, Germany) containing ethidium bromide and photographed.

**Human hematopoietic cytokines.** Specific activity (sp act) of cytokines was as follows: (1) rhIL-1α: sp act greater than 2 × 10⁸ U/mg protein determined by the antiproliferative effects on A375 indicator cells (Genzyme, Boston, MA); (2) rhIL-3: sp act greater than 2 × 10⁷ U/mg protein determined by the proliferative effect on TF-1 responder cells (Genzyme); (3) rhIL-6: sp act greater than 10⁸ U/mg protein determined by the amount required to induce proliferation of B9 hybridoma cells in vitro (Genzyme); (4) rhIL-11: sp act greater than 2 × 10⁷ U/mg protein determined by that amount required to induce half-maximum proliferation of T1165 murine hybridoma cells (Genzyme); and (5) rhGM-CSF: sp act greater than 1.25 × 10⁸ U/mg protein determined by a proliferation assay measuring TF-1 cell incorporation of 3H-TdR in a 96-hour microassay (Genzyme).

Human hematopoietic cytokine-neutralizing antisera and antibodies. Antisera and antibodies were as follows: (1) rabbit polyclonal antiserum to human IL-1α, 90% IgG: 1.0 mg of antibody neutralizes 1,000 U of rhIL-1α, as determined by the phytohemagglutinin-induced thymocyte proliferation assay (Genzyme); (2) rabbit polyclonal antiserum to human IL-3: 5 μg neutralizes 1,000 U rhIL-3; (3) rabbit polyclonal antiserum to human IL-6, 80% IgG: 10 to 50 μL/mL of antibody is recommended for the precipitation of rhIL-6; and (4) rabbit polyclonal antiserum to human GM-CSF, 80% IgG: 1.0 μg of antiserum neutralizes 20.0 ng of human GM-CSF as determined by radioimmunoassay based on anti-GM-C/SM-GM-CSF binding (Genzyme). The polyclonal antisera were able to bind to Staphylococcus protein A, as could be shown in a modified protein dot blot test using protein A gold (Biotrend, Cologne, Germany).

**Test conditions.** Cells were either used for the RHPA immediately or incubated in rolling plastic flasks for 8 hours in the presence of the above-mentioned mediators (10 ng/mL rhIL-1α, 10 pg/mL rhGM-CSF, 5 ng/mL rhIL-3, 10 ng/mL rhIL-6, and 10 ng/mL rhIL-11) in megakaryocyte medium.37

**RHPA.** We used the RHPA according to modifications described previously.34,36 Principles of this method are schematically shown in Fig 1. Briefly, freshly prepared stimulated or unstimulated human bone marrow cells were mixed with an equal volume of sheep red blood cells (SRBC) coupled to protein A (Sigma). Sheep leukocytes had been removed before conjugation procedure using a Percoll gradient method. The cell mixture was aliquoted into poly-L-lysine-coated Cunningham chambers and allowed to settle onto the glass slides for 60 minutes at 37°C in a humidified atmosphere of 5% CO₂ in air. To obtain a confluent layer of cells attached to the glass floor of the chamber, the excess of unattached cells was removed by rinsing each chamber with prewarmed RPMI 1640 medium containing 0.1% BSA, penicillin (100 U/mL), and streptomycin (100 μg/mL). The chambers were then filled with test solutions of 1/200 (vol/vol) dilutions of one of the above-mentioned polyclonal rabbit antisera, and slides were incubated at 37°C for 6 hours. Afterwards, chambers were washed with medium to remove any unbound antibody or secretory product, and chambers were filled with a dilution of biotinylated mouse anti-human monoclonal antiserum to human GM-CSF (GIBCO) to initiate plate formation by hemolysis (Figs 1 and 2A and B). After 30 minutes cells were exposed to 0.5% (vol/vol) glutaraldehyde in TRIS-buffered saline (TBS) for 3 minutes. After this fixation step, cover slips were removed from the chambers, and the slides were rinsed in TBS and prepared for immunolabeling.

**Identification of megakaryocytes.** Megakaryocytes were identified by immunostaining with a monoclonal antibody (MoAb) directed against platelet glycoprotein IIIa (Y2/51, CD61)40 according to the ABC method (Fig 2A and B).40 The MoAb was purchased from Dako.

**Quantitative analysis.** Data are presented as means ± SEM. Statistical analysis of the data was performed by the Mann-Whitney U test. In a pilot study a statistical analysis of variance between the different donors has been performed to assure reliability of data. However, no significant differences could be calculated, and for this reason, pooled data are presented.

**Controls.** Megakaryocyte viability was checked by the fluorescein diacetate method (Sigma).42,43 Cells were incubated with the CD61 for 10 minutes at 0°C. Subsequently, the cells were washed and then incubated with a 1:20 dilution of rhodamine-conjugated
MEGAKARYOCYTE CYTOKINE SECRETION

Fig 1. Schematic presentation of the RHPA performed on megakaryocytes. (I) Sheep erythrocytes (E) are coupled with protein A. (II) Addition of rabbit antisera against growth factors. (III) Cytokine release of the CD61-immunostained megakaryocytes. (IV) Addition of guinea pig complement generates a radial hemolytic area (halo formation) around the actively growth factor-secreting megakaryocytes with surrounding erythrocyte ghosts.

Fig 2. Photomicrographs of IL-3-secreting, CD61-immunolabeled small (A) and medium-sized (B) megakaryocytes at the center of a hemolytic plaque (halo) surrounded by ghosts of erythrocytes after 8 hours of exposure to 5 ng/mL rhl-L-3; original magnification × 870.

got F(ab')_2 antimouse IgG (Tago Inc, Burlingame, CA) and a fluorescein diacetate solution (0.1 µg/mL) for 20 minutes at 0°C. After washing, the cells were examined with a Zeiss Photoscope 3 (Carl Zeiss, Oberkochen, Germany) for both fluorescein and rhodamine fluorescence. The data presented are given in relation to the viable megakaryocyte population. In control experiments, the specificity of plaque formation was determined for the secretion of the examined cytokines by different means: plaque formation was not found if (1) human marrow cells were discarded, and the assay was performed using SRBC alone; (2) the specific antibodies were replaced by normal rabbit serum; (3) complement was omitted; or (4) SRBC not coated by protein A were used.

RESULTS

After centrifugation over a single-density Percoll gradient, the megakaryocyte cell fraction showed an average purity of 8% to 15%, whereas separation by the MACS technology achieved a concentration of 60% to 85%. Cytospin prepara-
of the nonviable cells increased to up to 30% after stimulation with the growth factors, irrespective of plaque formation.

The effects of stimulation (8 hours) by rhIL-3 (5 ng/mL) and rhIL-11 (10 ng/mL) on the secretory activity (IL-1α, IL-3, IL-6, and GM-CSF) of human megakaryocytes are shown in Figs 2A and B and 3A in comparison with the basal (spontaneous) activity. Data concerning stimulation with other cytokines, i.e., rhIL-1α (10 ng/mL), rhIL-6 (10 ng/mL), or rhGM-CSF (10 pg/mL), are summarized in Fig 3B. In all samples under study, cytokine release could be clearly determined by the ability of the immunostained megakaryocytes to form distinctive hemolytic zones in the plaque assay.

Spontaneous secretion of the tested growth substances by megakaryocytes in serum-free medium was low and ranged between 0.8 (IL-6) and 4.7 (GM-CSF) plaque-forming cells per 1,000 megakaryocytes (Fig 3A). After incubation for 8 hours before performing the RHPA, rhIL-3 and rhIL-11—but not rhIL-1α, rhIL-6, or rhGM-CSF—were capable of inducing a significant (P < .05) increase in the release of IL-6 in relation to the spontaneous secretion of this cytokine. In addition, stimulation with rhIL-3 was found to significantly (P < .05) enhance IL-3 (4.8 plaque-forming cells per 1,000 megakaryocytes) and GM-CSF secretion (59.6 plaque-forming cells per 1,000 megakaryocytes). After the observation of a stimulatory activity of rhIL-3 on human megakaryocytes in the RHPA, additional experiments using the RT-PCR were performed. This technique enabled us to investigate the behavior of cytokines in the absence of commercially available antibodies and to confirm that active synthesis was taking place by the cells under study. As shown in Fig 4, stimulation of a highly enriched megakaryocyte fraction with rhIL-3 for 8 hours markedly induced the expression of IL-6 mRNA, whereas no change in the expression of IL-6R, IL-9, IL-10, IL-12, and IL-13 mRNA could be observed. Treatment with IL-1α and IL-6 did not induce an expression of the examined cytokines. In control experiments with native bone marrow cell preparations, IL-6 mRNA expression could only be shown after stimulation with rhIL-1 and also rhIL-6.

With our test conditions, significant differences (P < .05) in the IL-6 and GM-CSF secretion of the megakaryocytes could be detected between the different stimulation protocols, especially concerning the influence of cytokines with overlapping activity spectrum (IL-6 and IL-11). It became apparent that the most actively secreting megakaryopoietic cells consisted predominantly of small, more immature elements (pro- and megakaryoblasts), although some release of cytokines was also measurable in the larger (polyploid) megakaryocytes.

**DISCUSSION**

As megakaryocytes belong to the most fragile cell population within the bone marrow, all enrichment procedures are prone to damage their morphology and alter their function. The problematic purification of megakaryocytes is bypassed by using the RHPA. This immunologic technique was originally developed by Jerne and modified by Molinaro and Dray to detect antigen formation of individual cells. Further improvements, which considerably facilitated this method, concerned the coupling of Staphylococcus protein A (SpA) instead of IgG to sheep erythrocytes. Antigens secreted by cells are bound by the antibodies to form immunocomplexes in the vicinity of the cell. These antigen-antibody complexes are bound by the SpA present on neighboring sheep erythrocytes with high affinity. Because the interaction of immunocomplexes with SpA does not interfere with complement binding, complement-induced lysis of the erythrocytes can occur. Thus, hemolytic plaques are formed surrounding antigen-secreting cells. Other investigators have described the diameter of the hemolytic plaques as a function of the amount of secreted product and have shown that as little as 10⁻¹⁸ mol/L of secreted antigen was detectable by the formation of hemolytic plaques.

This technique is ideally suited for the investigation of cytokine secretion in heterogeneous cell populations, as cytokine release is shown at the single-cell level. After detection of cytokine-secreting cells, immunocytochemistry with lineage-specific MoAbs is performed, and only immunolabeled cells are evaluated. Thus, we were able to evaluate the cytokine production by CD61⁺ human megakaryocytes in enriched and unpurified cell preparations. With this method, it was possible to examine at the single-cell level the secretory activity of immunoidentified human megakaryocytes.
and the effects of several cytokines (rhIL-1α, rhIL-3, rhIL-6, rhIL-11, and rhGM-CSF) on their secretory pattern. In contrast with the more commonly used reverse enzyme-linked immunospot (RELISPOT) technique, this method facilitates the clear-cut identification of each secretory active cell. According to our findings, the spontaneous secretion of the above-mentioned cytokines by CD61+ cells was low. This observation is in agreement with that of other investigators who did not detect larger amounts of secreted IL-6 by murine hematopoietic progenitors of the bone marrow. In comparison with the spontaneous secretion rate, rhIL-1α, rhIL-6, and rhGM-CSF did not induce a significant enhancement of cytokine release by megakaryocytes. On the other hand, optimal concentrations of both rhIL-3 and rhIL-11 exerted significant effects on IL-6 secretion. In addition, a significant increase in the release of IL-3 and GM-CSF, but not IL-1α, could be appreciated after stimulation with rhIL-3. For the first time, our results prove the release of IL-1α, IL-3, IL-6, and GM-CSF by single normal human megakaryocytes.

Perhaps the most compelling evidence supporting the assumption of an endogenous cytokine production was that the RT-PCR showed measurable amounts of IL-6 mRNA only after stimulation of a highly enriched megakaryocyte population with rhIL-3. In control experiments on unpurified bone marrow cell preparations, we tested the influence of rhIL-1α and rhIL-6 on cytokine expression. As the induction of IL-6 mRNA by rhIL-3 stimulation was only observed in the enriched megakaryocyte fraction and not in unpurified bone marrow cells, other contaminating cell lineages could be excluded as the source of IL-6. On the other hand, IL-6 mRNA could be shown after treatment of the control cell population with IL-1α. Using the RT-PCR, we did not show an expression of a battery of cytokines (IL-9, IL-10, IL-12, IL-13, and IL-6R). We were not able to examine the secretion of these cytokines, because no polyclonal antibodies were available commercially at the time of our studies. This is in contrast with the findings of a former study. However, test conditions differed considerably because those investigators examined megakaryocytes previously cultured for at least 6 days in serum-containing medium. Also, the enrichment procedure was not as effective as in our study, so that other contaminating cell lineages cannot be ruled out as the source of this cytokine.

Many in vivo and in vitro investigations have clearly established the influence of several cytokines on proliferation and maturation of human megakaryopoietic progenitors. The most effective cytokine to support megakaryocyte proliferation and also differentiation is known to be IL-3, although there is some evidence that this cytokine is not very effective in promoting endoreduplication. IL-11 and IL-6 were reported to display a wide spectrum of biologic activities. Similar to IL-6, IL-11 acts synergistically with IL-3 to shorten the G0 period of the cell cycle, particularly in early progenitor cells. However, the effect of GM-CSF on megakaryocyte progenitors continues to be controversial. Although there may be no doubt about the involvement of several cytokines in the regulation of human megakaryopoiesis, the active role of this cell lineage concerning the

Fig 4. RT-PCR of human megakaryocytes. Human megakaryocytes were separated from sternal bone marrow and cultured for 8 hours at a concentration of 10⁵ cells per milliliter in RPMI 1640 medium supplemented with 0.1% BSA. Samples were stimulated by the addition of 5 ng/mL rhIL-1, 5 ng/mL rhIL-3, or 5 ng/mL rhIL-6, respectively, to the serum-free tissue culture medium. After collection of the cells by centrifugation, cellular mRNA was isolated and reverse transcribed. The cDNA was split and subjected to PCR amplification using primers for β-actin (act), IL-6, IL-6R, IL-9, IL-10, IL-12, and IL-13. Representative results of one experiment are shown. On the left side, results are shown for the unpurified bone marrow populations. Stimulation with rhIL-1 and also rhIL-6 induces mRNA for IL-6. In comparison, an induction of IL-6 mRNA can be appreciated in the enriched megakaryocyte fraction (right) only after stimulation with rhIL-3.
synthesis and secretion of these mediators is virtually unknown. Most frequently, bone marrow fibroblasts and other nonhematopoietic or stroma cells have been suggested to provide the main source for production and release of these mediators, whereas megakaryocytes were regarded as acting merely as passive target cells. This assumption is caused by the fact that, so far, none of the examined cytokines have been shown to originate from the megakaryopoietic lineage. Navarro et al have taken a different view because they were able to show the expression and synthesis of IL-6 and IL-6R by human megakaryocytes.

Because of the difficulties associated with the enrichment and isolation procedures of megakaryocytes, other investigators have preferred to study cytokine expression and protein synthesis in various megakaryocytic cell lines. Under these circumstances, the investigators succeeded in showing the secretion of IL-6, GM-CSF, and IL-1 in the culture supernatant after stimulation with phorbol myristate acetate (PMA) using the enzyme-linked immunosorbent assay (ELISA) technique.

Our results, particularly concerning IL-3, provide convincing arguments to support the hypothesis that megakaryopoiesis is controlled by autocrine and paracrine feedback mechanisms modulated by various mediators. Moreover, cytokines produced by megakaryocytes could act on other hematopoietic elements, as well as modulate the proliferation and function of stromal cells within the bone marrow microenvironment. The RHPA in combination with the RT-PCR technique has proven to be an excellent tool to clarify the various interrelations in the human bone marrow microenvironment.

REFERENCES

29. Jerne NK, Henry C, Nordin AA, Fuji H, Koros AMC, Lefko-
42. Rotman B, Papermaster BM: Membrane properties on living mammalian cells as studied by enzymatic hydrosis of fluorogenic esters. Proc Natl Acad Sci USA 55:134, 1966
45. Lewis CE: Detecting cytokine production at the single-cell level. Cytokine 3:184, 1990
Secretion of cytokines (interleukins-1 alpha, -3, and -6 and granulocyte-macrophage colony-stimulating factor) by normal human bone marrow megakaryocytes

C Wickenhauser, J Lorenzen, J Thiele, A Hillienhof, K Jungheim, B Schmitz, ML Hansmann and R Fischer