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Cytokine release by human bone marrow cells: analysis at the single cell level

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Abstract Regulation of haemopoiesis is closely mediated by a number of growth factors in the marrow microenvironment. The identification of the cell type secreting these regulatory polypeptides is difficult due to the heterogeneity of bone marrow cells. To analyse the release of haemopoietic growth factors by normal human bone marrow cells at the single cell level, we employed the reverse haemolytic plaque assay (RHPA). Freshly isolated human marrow cells were examined for the release of interleukin-1α (IL-1α), IL-3, IL-6 and granulocyte-monocyte colony stimulating factor (GM-CSF). In order to identify various cytokine-secreting cell types, the RHPA was combined with immunocytochemical or enzymatic staining. The total of secreting marrow cells as well as the amount of several secretory haemopoietic subpopulations could be determined with this technique under various conditions. Following incubation with pure serum-free medium without addition of any mediator, only few cells secreting either IL-1α, IL-3, IL-6 or GM-CSF could be observed. After 2 h incubation with recombinant human-IL-1α (rhIL-1α) (10.0 ng/ml) or rhGM-CSF (10.0 pg/ml) the number of cytokine-secreting cells significantly increased for all secretory products tested. Using cytochemical staining reactions, we were able to identify 55% of all cells secreting a specific cytokine. Glycophorin C-positive erythropoietic cells turned out to be the largest fraction (up to 89%) of cytokine-releasing haemopoietic cells, followed by neutrophil granulocytes (between 6 and 48%), and monocytes/macrophages (between 4 and 23%). Only few CD 61-positive cytokine-secreting megakaryocytes could be detected. Dose- and time-dependent kinetics after stimulation with rhGM-CSF revealed that the bulk of secretory activity originates from haemopoietic or rather from erythropoietic cells following low level stimulation and after short stimulation time. Thus, our data are in keeping with the assumption, that especially erythropoietic cells are producing a repertoire of cytokines that is thought to exhibit regulatory functions within marrow microenvironment. In the present study the RHPA is presented as an appropriate tool for measuring cytokine release not only of cells of the haematopoietic system but also of other tissues, for example solid tumours or malignant lymphomas.

Key words Cytokine release · Granulocytes
Erythroid precursors · Reverse haemolytic plaque assay
Human bone marrow

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Introduction

The role of growth factors regulating proliferation and differentiation of haemopoietic cells has been amply demonstrated both in vitro (Heyworth et al. 1990) and in vivo (Moore 1991; Rowe and Rapoport 1992). Glycoproteins such as interleukin-3 (IL-3), IL-6, granulocyte-monocyte colony stimulating factor (GM-CSF), and IL-1 are either acting directly on distinct haemopoietic subpopulations (Sieff et al. 1985; Briddell and Hoffman 1990; Otsuka et al. 1991) or in combination with other growth factors (Heyworth et al. 1988; Ikebuchi et al. 1988; Carcacciolo et al. 1989), thus contributing to regulatory cytokine networks (Broxmeyer 1986; Greenberger 1991) that ensure adequate haemopoiesis. There is evidence that release of these growth factors can be induced by various marrow cell types including haemopoietic cells such as T cells, neutrophil granulocytes and monocytes (Brach and Herrmann 1991) as well as by the adherent layer of long-term bone marrow culture (Quesenberry 1989) comprising fibroblasts, macrophages, and endothelial cells (Dorshkind 1990; Eaves et al. 1991; Kittler et al. 1992). To date, hardly any information exists regarding the secretory activity of the different bone marrow subpopulations and the quantitative differences in secretion amongst the various cell types. This lack of knowledge is largely related to the fact that investiga-

tions concerning cytokine production are generally based on bio- and immunoassays which determine accumulated amounts of proteins released into culture supernatants by entire cell populations.

To visualize and to measure the release of IL-1α, IL-3, IL-6, and GM-CSF by single marrow cells, we have employed the reverse haemolytic plaque assay (RHPA; Jerne et al. 1974; Molinaro and Dray 1974), which facilitates the determination of actively secreting cells in a mixed cell population (Lewis et al. 1989, 1990). Since each of the cytokines is secreted by more than one cell type, this assay was coupled with several staining procedures to identify different secreting haemopoietic subpopulations contributing to the total release of each cytokine.

In the present study, we have investigated the spontaneous cytokine release as well as the stimulatory effects of recombinant-human-IL-1α (rhIL-1α) and rhGM-CSF on the secretion of IL-1α, IL-3, IL-6, and GM-CSF in the entire marrow population. Amongst the total of secretory marrow cells we could identify considerable amounts of haemopoietic cell elements showing differential plaque formation under the given test conditions. Our data provide persuasive evidence that the RHPA represents an appropriate tool to evaluate precisely and to compare the conditions modifying secretory activity of particular marrow cell elements. Furthermore, this technique allows the demonstration of cytokine release by various other normal and pathological tissues.

Materials and methods

After informed consent had been obtained, sternal bone marrow specimens from 22 haematologically normal patients undergoing surgical thoracotomy were studied in the reverse haemolytic plaque assay.

Marrow particles were immediately transferred to serum-free RPMI 1640 medium (Gibco, Paisley, GB) containing penicillin (100 U/ml) and streptomycin (100 µg/ml; Biochrom, Berlin, FRG). Marrow cells were isolated from bone particles in 30 mm culture dishes (Greiner, Frickenhausen, FRG) after gentle agitation for 30 min at 37° C in a humidified atmosphere of 5% carbon dioxide in air. Cells were washed twice in medium and resuspended to a final concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 0.1% bovine serum albumin (BSA; Sigma, St. Louis, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Cells were either used for the RHPA immediately or incubated in rolling plastic flasks for 2 h in the presence of mediators such as 10 ng/ml rhIL-1α or 10 pg/ml rhGM-CSF (Genzyme, Boston, MA, USA). For dose-response tests rhGM-CSF was applied using concentrations of 0.1, 1.0, 10.0, and 50.0 pg/ml. Time-dependent stimulation with 10.0 pg/ml rhGM-CSF was performed for 2, 4, and 6 h, respectively.

The RHPA was performed according to the modifications described by Lewis et al. (1989). Freshly prepared stimulated or unstimulated human bone marrow cells were mixed with an equal volume of sheep red blood cells (SRBC) previously coupled to protein A (Sigma). Any sheep white blood cells had been removed prior to conjugation procedure using the Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) method. The cell mixture was aliquoted into Cunningham chambers and allowed to settle onto the glass slides for 45 min at 37° C in a humidified atmosphere of 5% carbon dioxide in air. To leave a confluent layer of cells attached to the glass floor of the chamber, excess unattached cells were removed by rinsing each chamber with warm 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 dilutions (v/v) (1/200) of rabbit antiserum raised against rhIL-1α, IL-3, IL-6, or GM-CSF (Genzyme, Boston, Mass., USA), and slides were incubated at 37° C for 6 h. Thereafter chambers were washed with medium to remove any unbound antibody or secretagogue, and chambers were filled with a dilution of 1/50 guinea-pig complement (Gibco, Paisley, GB) to initiate plaque formation. After 30 min cells were exposed to either 0.2% (v/v) trypan blue solution and incubated for 5 min at 37° C (to test the viability of the bone marrow cells) or 0.1% (v/v) glutaraldehyde in phosphate buffered saline (PBS) for 3 min. After fixation cover slips were removed from the chambers and the slides were either dried prior to enzymatic staining or rinsed in PBS and prepared for immunolabelling.

For quantitative analysis each complete chamber of cells was systematically scanned using a Leitz (Wetzlar, FRG) forward-projection microscope, and the number of plaque-forming cells as well as the area of haemolytic plaques formed under each test condition was recorded applying an image-analysis package devised for the Apple-Macintosh by Dr. J. Lorenzen. Data are presented as means±standard error of mean. Statistical analysis of the data was performed by the Mann-Whitney U test.

At the end of the RHPA cells were either stained by routine enzymatic procedures or by immunolabelling according to the alkaline phosphatase anti-alkaline phosphatase method (Cordell et al. 1984). Erythropoietic progenitor cells were recognized by the mouse monoclonal antibody (mAb) Ret40f (Gatter et al. 1988), megakaryopoietic cells and thrombocytes by CD 61 (Gatter et al. 1988), monocytes/macrophages were identified either by the mAb KiM1P, courtesy of M. R. Parwaresch, Kiel (Radzun et al. 1991) or by the positive reaction for the 'unspecific esterase', elements of the neutrophil granulopoiesis were determined by the naphthol-AS-D-chloroacetate esterase reaction. Lymphocytes were immunostained using CD 45 RA. The mAb Ret40f and CD 61 were purchased from Dako, Hamburg, FRG, CD 45 RA from Biotest, Dreieich, FRG.

The trypan blue exclusion test indicates cell viability and demonstrated that in our study >90% of human bone marrow cells were viable at the end of 6 h in the RHPA. The data presented here, are exclusively regarding the viable marrow cells.

Specificity of plaque formation was determined for the secretion of IL-1α, IL-3, IL-6, and GM-CSF: plaque formation was not visible if (a) human marrow cells were omitted and the assay was performed using sheep red blood cells alone, (b) the specific antibodies were omitted and replaced by normal rabbit serum, (c) complement was omitted or (d) sheep red blood cells not coated with protein A were used.

About 70% of plaque-forming cells also revealed cell associated IL-1α, IL-3, IL-6, or GM-CSF as determined by using the antibodies mentioned above for immunolabelling.

Regular composition of bone marrow was controlled by cytospin preparations of the isolated marrow cells. At the end of the RHPA test chambers showed a similar cellular composition as the cytospin preparations except members of the megakaryopoiesis which were severely depleted.

Results

Following resuspension of freshly isolated marrow cells in pure RPMI 1640 medium and subsequent transfer into the test chambers, spontaneous release of IL-1α, IL-3, IL-6, and GM-CSF was demonstrable. Cytokine production measured by the number of plaque-forming cells (Fig. 1) ranged between 20 and 30 cytokine-secreting cells per 1.5×10^4 bone marrow cells (Figs. 2a, b; 3).

Addition of rhIL-1α (10.0 ng/ml) or rhGM-CSF (10.0 pg/ml) for 2 h prior to the RHPA significantly increased ($p<0.05$) the overall number of secretory cells for all cytokines tested. Stimulatory conditions as chosen here we-

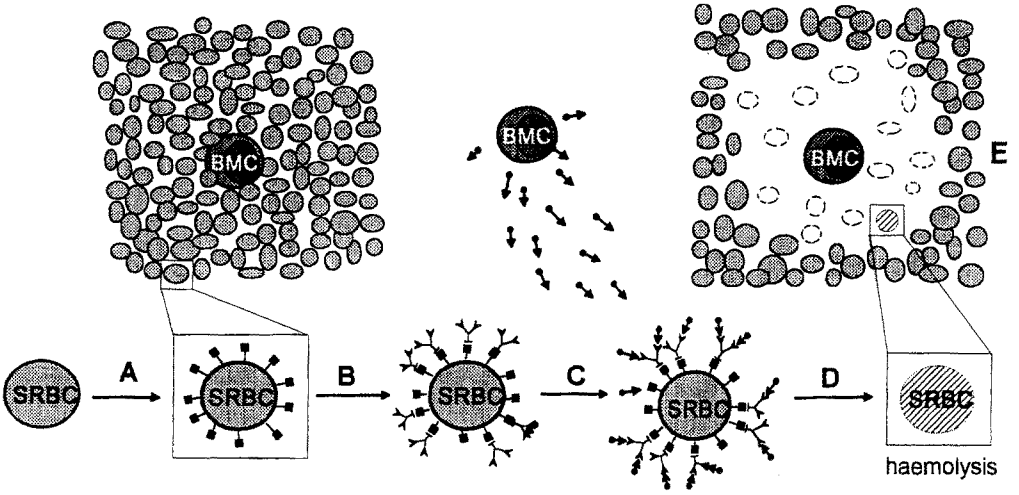

Fig. 1 Schematic presentation of the reverse haemolytic plaque assay (RHPA). **A** Sheep red blood cells (SRBC) are coupled to protein A. **B** Addition of rabbit antisera against IL-1α, IL-3, IL-6, and GM-CSF. **C** Cellular secretion of the cytokines by bone marrow cells (BMC). **D** Addition of guinea-pig complement. **E** Radial haemolytic area (halo formation) around the secreting BMC

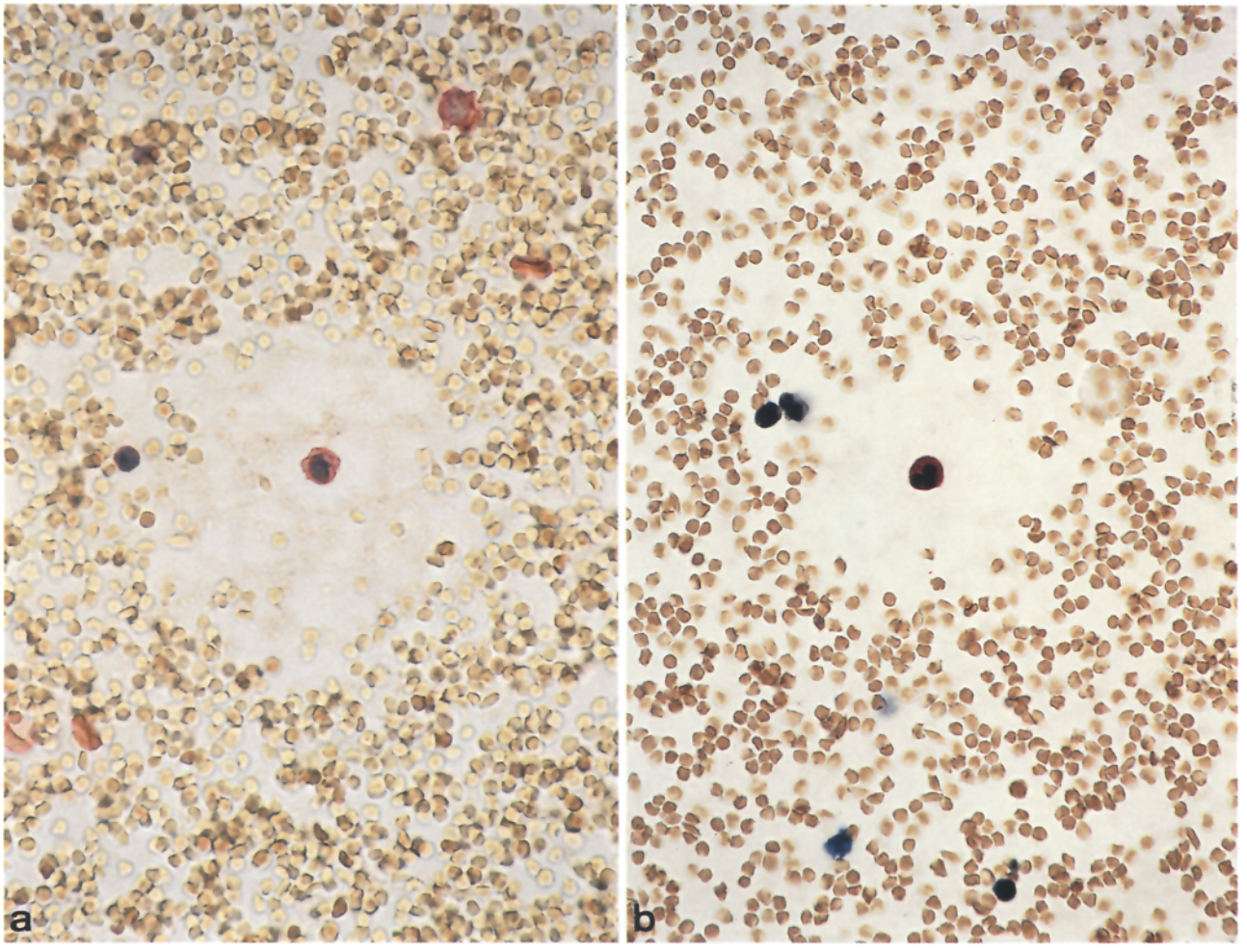

Fig. 2a, b Photomicrograph of two cytokine-secreting haemopoietic cells after 2 h of exposure to 10 pg/ml rhGM-CSF. **a** An erythropoietic cell immunolabelled with the monoclonal antibody Ret40f at the centre of an IL-1α plaque. **b** An element of the neutrophilic granulopoiesis, determined by the naphthol-AS-D-chloroacetate esterase reaction after secretion of IL-6

re based on a pilot study on the influence of dose- and time-dependent kinetics.

Furthermore, the amounts of secreting haemopoietic cells which were identified by different staining procedures are indicated in Fig. 3: the number of secreting granulopoietic cells and monocytes/macrophages only moderately increased upon stimulation, whereas numbers of cytokine-releasing erythroid cells were consider-

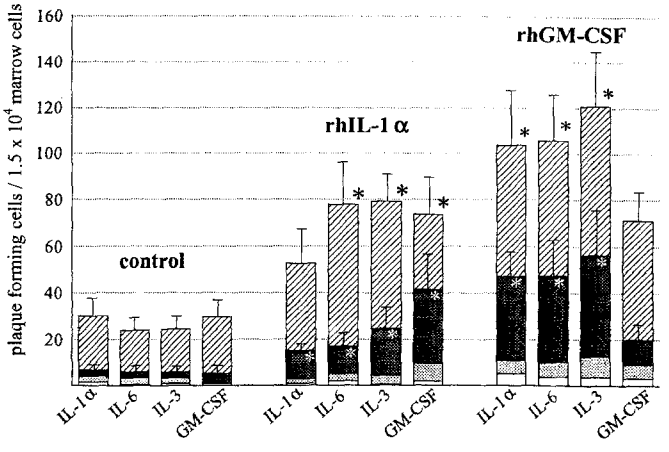

Fig. 3 Release of IL-1α, IL-3, IL-6, and GM-CSF by normal human bone marrow cells. Effect of no addition, 10.0 ng/ml rhIL-1α, and 10.0 pg/ml rhGM-CSF on plaque formation. Number of plaque forming cells (mean±SEM) per 1.5×10^4 marrow cells of pooled data from 12 separate experiments.* $p<0.05$ compared with the controls. Bars also indicate the amount of secretory monocytes/macrophages (□) granulopoietic (▩), and erythropoietic cells (■) as well as the secretory marrow cells (▨) not identified by the applied staining procedures

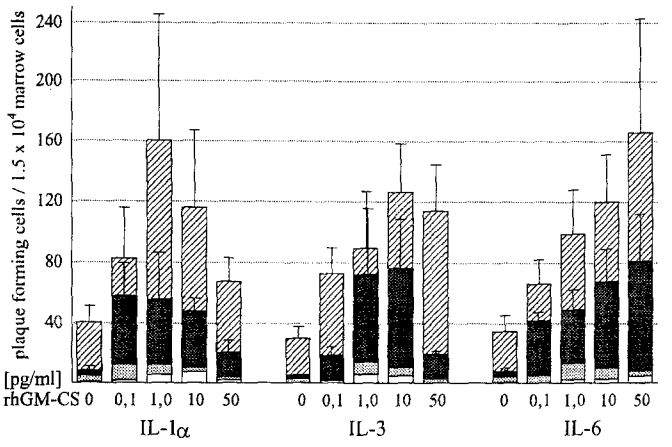

Fig. 5 Dose-dependent release of IL-1α, IL-3, and IL-6 by normal human bone marrow cells after addition of 0.1, 1.0, 10.0 and 50.0 pg/ml rhGM-CSF for 2 h. Number of plaque forming cells (mean±SEM) per 1.5×10^4 marrow cells of pooled data from 5 separate experiments. * $p<0.05$ compared with the controls. Bars also indicate the amount of secretory monocytes/macrophages (□), granulopoietic (▩), and erythropoietic cells (■) as well as the secretory marrow cells (▨) not identified by the applied staining procedures

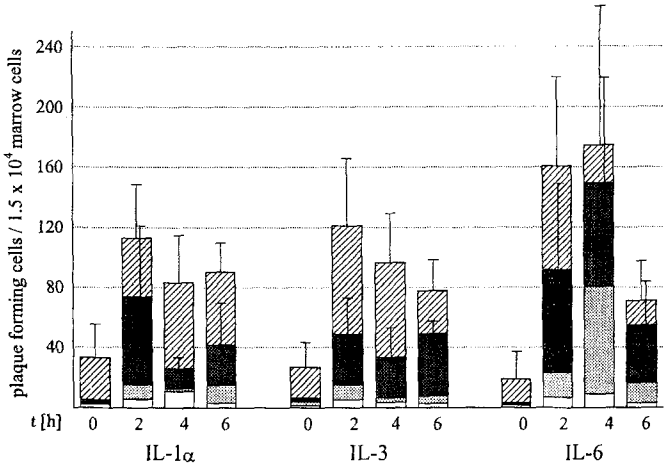

Fig. 4 Time-dependent release of IL-1α, IL-3, and IL-6 by normal human bone marrow cells after addition of 10.0 pg/ml rhGM-CSF for 2 h, 4 h, and 6 h compared with the control. Number of plaque forming cells (mean ±SEM) per 1.5×10^4 marrow cells of pooled data from 5 separate experiments. Bars also indicate the amount of secretory monocytes/macrophages (□), granulopoietic (▩), and erythropoietic (■) cells as well as the secretory marrow cells (▨) not identified by the applied staining procedures

ably enlarged. Induction of plaque formation in the haemopoietic cells was most effective after addition of rhGM-CSF for the release of IL-1α, IL-3, and IL-6 by erythroid cells. The amount of plaque-forming megakaryocytes is not specified, as there was a severe depletion of this cell lineage in the test chambers at the end of the RHPA. This effect was not observed with the other haemopoietic cell elements. Plaque formation by CD 45 RA-positive T-cells was not observed under the present experimental conditions.

Exposure of marrow cells to 10.0 pg/ml rhGM-CSF for 2 h, 4 h, and 6 h led to a peak of plaque formation at 2 h. At this time erythroid cells comprised the largest amount of secretory haemopoietic elements (Fig. 4). Further incubation periods had a moderate additional effect exclusively on the number of IL-6-releasing cells, which still increased after 4 h of incubation. This result was probably due to the large amount of IL-6-secreting granulopoietic cells which could be observed here.

When marrow cells were exposed to various concentrations of rhGM-CSF for 2 h, a dose-dependent induction of plaque formation could be noted (Fig. 5). The amounts of IL-1α-producing marrow cells resulted in a bell-shaped curve with a maximal stimulatory effect being exhibited by 1.0 pg/ml rhGM-CSF. The amounts of IL-3 and IL-6-releasing cells were elevated proportionally to rhGM-CSF concentrations and significant increases of secretory cells were induced by 10.0 pg/ml and 50.0 pg/ml rhGM-CSF. Amongst the cytokine-releasing haemopoietic elements the erythroid cells turned out to be the predominant cell type. As indicated in Fig. 5, erythroid cells were comprising the largest relative amount (between 60 and 70%) of the total of IL-1α, IL-3, and IL-6-secreting marrow elements following low doses (0.1 or 1.0 pg/ml) of rhGM-CSF.

The size of plaques formed by the different haemopoietic cell types was compared with the total of plaque-forming marrow cells for each of the cytokines tested. Spontaneous plaque formation ranged between 1293 and 1384 μm^2, while sizes of plaques increased remarkably after addition of rhGM-CSF. Evaluation of pooled data from all experiments performed after stimulation with rhGM-CSF yielded the following results: statistically the area of haemolytic plaques formed by IL-1α and IL-3-

releasing erythroid cells turned out to be significantly larger than by the other haemopoietic cell types identified (Fig. 6).

Discussion

Research on haemopoietic growth factors has elucidated biology and targets of modulatory molecules such as IL-1, IL-3, IL-6, and GM-CSF which regulate in part production and/or differentiation of specific lineages of blood cells (Heyworth et al. 1990). Recent progress in this field has implicated a central role of IL-1, IL-3, IL-6, and GM-CSF in megakaryopoiesis (Takahashi et al. 1991; Briddell et al. 1992). However, until now cellular sources of these secretion products in human bone marrow are not well defined. This may be due to the fact that procedures generally applied detect either cell-associated growth factors, or growth factor activity in culture supernatants, or specific mRNA (De Groote et al. 1992; Kittler et al. 1992). Altogether, these various techniques are suitable to disclose indirect evidence for secretory activity, but do not necessarily identify the nature of secretory cells. Only Shirai et al. (1993) have recently demonstrated the release of IL-6 by murine bone marrow cells applying the enzyme-linked immunospot method (Czerkinsky et al. 1983), which allows secretory cells to be enumerated and to be identified by fluorescence activated cell sorting.

The aim of this study was to present an immunoassay that permits measurement and visualization of cytokine release by individual bone marrow cells. Therefore, we have adapted the RHPA (Molinaro and Dray 1974; Lewis et al. 1990) for the detection of IL-1α, IL-3, IL-6, and GM-CSF released by freshly isolated normal human bone marrow cells under several test conditions. While spontaneous release of these haemopoietic growth factors was low, a statistically significant increase in the number of secreting cells could be obtained by minor stimulatory treatment with rhIL-1α or rhGM-CSF during 2 h (Figs. 2a, b; 3). The RHPA, being combined with several staining procedures, enabled us to identify different haemopoietic cell types comprising up to 55% of the total of secretory marrow cells: the largest fraction of actively secreting haemopoietic cells turned out to be Ret40f-positive erythroid precursor cell population (Figs. 2a, 3). In contrast the majority of monocytes/macrophages and neutrophils did not secrete detectable amounts of these cytokines, and immunoidentified T cells did not reveal any secretory activity under these conditions.

Erythroid progenitors, which are known to be target of a number of growth factors (Metcalf 1984; Whetton and Dexter 1989), are rarely demonstrated to produce any regulatory molecules by themselves (Sytkowski et al. 1990; Hermine et al. 1991). This might be due to the fact that methods measuring bulk of cytokine release in culture supernatants, are not as sensitive as the RHPA (Lewis et al. 1989; Lewis 1991) which allows cellular products of less than 10^{-18} M to be discerned at the sin

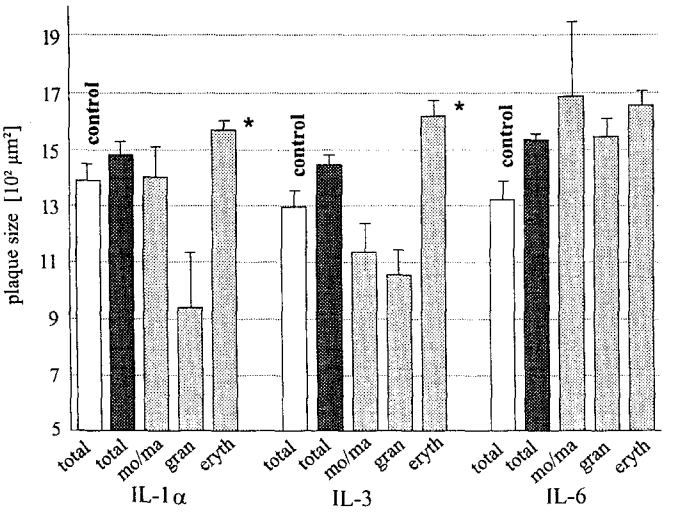

Fig. 6 Size of plaques (median±SEMed) formed by normal human bone marrow cells secreting IL-1α, IL-3, or IL-6 after addition of 10.0 pg/ml rhGM-CSF for 2 h compared with the control. Pooled data obtained from 22 separate experiments. * $p<0.05$ compared with the total and also with the different secretory haemopoietic cell types. Bars also indicate the median of plaque sizes formed by the total of secretory cells as well as by monocytes/macrophages (mo/ma), neutrophil granulopoietic (gran) and erythroid (eryth) cells

gle cell level. Moreover, cytokine production by erythroid cells possibly is a transient event that may be masked by predominant secretory activity of other marrow cell types under test conditions commonly applied. Detection of growth factor release is most often based on culture techniques that require incubation periods of at least 24 or 48 h, while the RHPA allows to determine the immediate secretory response. Our data, obtained by the RHPA, suggest that cytokine release by erythroid cells may be most effectively induced after addition of low doses of mediators and following short stimulation times. When studying the dose-dependent effect of rhGM-CSF on the release of IL-1α, IL-3, and IL-6 the relative amount of secreting erythroid cells was maximal (between 50 and 75%) at low doses (0.1 and 10.0 pg/ml) of rhGM-CSF. Furthermore, addition of rhGM-CSF induced maximal effect in IL-1α and IL-6-releasing erythroid cells after incubation periods of 2 h. However, larger doses and longer incubation periods increased the relative amount of cytokine producing 'non-haemopoietic' cells, that is, cells that were not identified by our staining procedures (specific for haemopoietic cell types). Moreover, sizes of plaques formed by IL-1α and IL-3-releasing erythroid cells are significantly differing from those formed by the total of secretory marrow cells or by the other haemopoietic elements. These findings clearly indicate erythroid cells to be significantly secretors of cytokines under these conditions (Fig. 6).

After addition of rhGM-CSF a further time-dependent effect could be observed (Fig. 4): incubation periods of 4 h or 6 h resulted in a remarkable decrease in the number of IL-1α, IL-3, and IL-6-secreting cells. This result was

probably related to a short-lived stimulatory effect of 10.0 pg rhGM-CSF/ml on the activity of secretory cells that may be degraded by cell catabolism as well as by induction of inhibitory molecules. The only additional effect of longer lasting stimulation with rhGM-CSF was exerted on the number of IL-6-releasing marrow cells after 4 h of incubation. It was also observed that in this case a large number of IL-6 secreting granulopoietic cells were found (Fig. 4), in agreement with the results of Cicco et al. (1990), who reported specific IL-6 mRNA in cell lysates of polymorphonuclear neutrophils and detected IL-6 protein in culture supernatants after addition of rhGM-CSF and an incubation period of 24 h. However, the RHPA allows us to confine the duration of incubation period to 4 h. In our case it could be demonstrated that the number of IL-6-releasing granulopoietic cells was smaller at 2 h than at 6 h after stimulation with rhGM-CSF.

In conclusion, our data reveal that the RHPA is an appropriate tool to elaborate the differential conditions that are required by distinct bone marrow cell types for cytokine release. The results of these preliminary experiments are in keeping with the assumption that erythroid progenitor cells, which have been neglected in terms of their possibly active role in bone marrow function, have the capacity to secrete a number of growth factors that may regulate haemopoiesis in either an autocrine or paracrine manner.

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