Leukemia (1997) 11, 1700–1710

Flow cytometry measurement of GM-CSF receptors in acute leukemic blasts, and normal hemopoietic cells

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A quantitative analysis of expression levels of GM-CSF receptors was performed by flow cytometry in different disease categories, ie AML (n = 72), ALL (n = 18), and MDS (n = 12), as well as 12 healthy volunteers, using three different unconjugated GM-CSF/R monoclonal antibodies (McAbs) (HGM-CSFR (CD116), M5D12, 4B5F5), and appropriate standards. By using the reference HGM-CSFR McAb, in healthy subjects we found detectable levels of GM-CSF/R on blood monocytes (mean MESF (molecules of equivalent soluble fluorochrome)/cell: 36.1 × 10^3), neutrophils (mean MESF/cell: 7.4 × 10^3), bone marrow (BM) myelo-monocytic precursors (MESF range for the myeloid component, ie promyelocytes, myelocytes, metamyelocytes: 11.7–40.5 × 10^3, and for the monocytic lineage: 25.7–69.2 × 10^3), and in two distinct subsets of BM CD34$^+$ progenitor cells (GM-CSF/R dim: 2.5 × 10^3 MESF/cell, GM-CSF/R bright (10% of the total number of CD34 cells: 22.0 × 10^3 MESF/cell). In these subjects, there was no correlation between the expression levels of GM-CSF/R and CFU (CFU-GM, CFU-GEMM, BFU-E) colony production. Among the AML samples, M5D12 McAb was positive in 33%, 4B5F5 McAb in 90%, and HGM-CSF/R McAb in 78% of the cases examined (range of MESF/cell for the HGM-CSFR McAb: 0.9 × 10^3–106.7 × 10^3). The highest MESF values were seen in the M5 FAB subvariety (mean: 39.4 × 10^3), where all the patients tested (n = 20) showed a strong positivity for the HGM-CSFR McAb. On the contrary, all ALL samples were GM-CSF/R negative except in two patients, who displayed a dim GM-CSF/R positivity (My+ALL: 1.3 × 10^3 MESF/cell; pro-B ALL: 1.0 × 10^3 MESF/cell). In most (>70%) M1 FAB subtypes, GM-CSF/R+ blasts co-expressed CD34^{low}, HLA-DR^{high}, CD33, CD38 antigens, and had little or no capacity to form CFU-GM colonies. GM-CSF/R+ blasts from the M5 FAB category were also positive for CD14, CD11c, CD33 and CD87. Furthermore, the number of GM-CSF/R expressed by leukemic cells from five out of 72 (7%) AML patients was above the highest values seen in normal samples (>69.2 × 10^3 MESF/cell), allowing the possibility of using this marker for the monitoring of the minimal residual disease (MRD) in a subset of AML. Cell culture studies aimed at evaluating GM-CSF receptor modulation following AML blast exposure to rhGM-CSF showed two distinct patterns of response; in the first group (6/10 cases) rhGM-CSF down-modulated GM-CSF receptors, whereas in the second group (4/10 cases), rhGM-CSF treatment was associated with either an increase or no change in the number of GM-CSF/R. In conclusion, cellular GM-CSF/R expression was variable and ranged from undetectable (ALL and a minority of AML) to very high intensities in M5 AML, and were also documented in some M0 AML, thus suggesting the concept that GM-CSF/R detection may be of help in lineage assignment of undifferentiated forms. Since the number of GM-CSF/R on AML blasts may be modulated after GM-CSF treatment, it can be postulated that the clinical use of GM-CSF in this disease may be optimized by a dynamic analysis of the number and the affinity status of GM-CSF-R in blasts and normal hemopoietic cells.

Keywords: GM-CSF receptor; flow cytometry; acute myeloid leukemia; GM-CSF; receptor modulation

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Received 17 February 1997; accepted 6 June 1997

Introduction

In the last few years, the molecular cloning and purification of several hemopoietic growth factors (GF) and cytokines, such as the granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), erythropoietin (EPO), thrombopoietin (TPO), interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6 and IL-12 (among others), has made their use (at least for some of these GF) possible in the clinical setting. Apart from EPO, G-CSF and GM-CSF are the most widely used colony-stimulating factors for the treatment of hematological malignancies.[1–4]

The functions and principal biologic properties of GM-CSF have been extensively studied, allowing a better understanding of the clinical role played by this molecule in the lymphohemopoietic system. GM-CSF is a multilineage hemopoietic growth factor which stimulates proliferation, differentiation and survival of progenitor cells, enhances the functional activities of mature myeloid effector cells (monocytes and granulocytes), and plays a key role in host defense and in the inflammatory process.[1–5]

The main clinical use of rhGM-CSF (recombinant human GM-CSF) is to stimulate recovery of neutropenia and/or monocytopenia following myeloablative chemotherapy, radiotherapy, and/or bone marrow transplantation. Although the administration of rhGM-CSF in patients affected by lymphoid malignancies (ie malignant lymphomas, multiple myeloma, Hodgkin's disease, acute lymphoblastic leukemias) is widely accepted, its clinical usefulness and safety in the management of acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) is still controversial. Nevertheless, a number of reports have shown that rhGM-CSF could be given to AML patients without increasing remission or relapse rates, or shortening the life expectancy of the disease.[6–11] On the basis of these data, it has been postulated that the risk of stimulating the leukemia growth following GM-CSF therapy is low, even in patients with myeloid malignancies.[2,6–11] The schedules so far adopted for the *in vivo* use of rhGM-CSF in AML are as follows: (1) post- and/or during chemotherapy;[6–12] (2) pre- and/or during chemotherapy;[13–21] (3) pre- and post-chemotherapy;[22,23] (4) as mobilising agents to induce the release of progenitor cells from the bone marrow into the circulation (peripheral blood progenitor cells transplantation (PBPC).[24,25] Concerning the use of GM-CSF before chemotherapy, it has been reported that this treatment may induce a recruitment of quiescent (G0) blast cells into active cell cycle phases (S-G2+M). In this way, more vulnerable S-phase blast cells can easily be killed by cell cycle-specific cytostatic drugs.[26–28]

The biologic activity of GM-CSF on hemopoietic cells depends upon its binding to specific receptors, which may be expressed on both leukemic and normal cells.[29–33] The receptor for GM-CSF is a high affinity receptor composed of alpha and beta subunits with molecular weights of 80 and 130 kDa, respectively. The alpha subunit binds GM-CSF with low affinity; the beta subunit (common to interleukin 3 and 5 receptors)

does not bind GM-CSF, but its association with the alpha-subunit generates high affinity receptors for GM-CSF. At the 5th International Workshop on Leukocyte Differentiation Antigens held in Boston, MA, USA, in November 1993, this molecule was termed CDw116.[30,33] The CDw (w stands for workshop) designation has been given to this receptor as its reactivity has not yet been completely defined, and therefore will have to be re-evaluated in future workshops. Nevertheless, extensive multidisciplinary studies which included flow cytometry, biochemical, molecular, histochemical and serologic analysis have allowed the identification of one McAb specific for the GM-CSF receptor (HGM-CSFR). Other McAbs, although giving rise to conflicting results when evaluating McAbs reactivity with cells transfected with CD116 cDNA and binding to GM-CSF protein, showed a pattern of reactivity rather similar to that of the reference HGM-CSFR McAb.[30] The recent availability of quantitative microbead calibration standards has allowed a precise, reproducible cytofluorimetric quantitation of the number of receptors per cell exhibited by cell populations labeled with monoclonal antibodies directed against different cytokine receptors.[34] The microbeads are to be considered reference standards which allow the comparison between the fluorescence intensity showed by cells stained with a given McAb and that of the four- or five-bead populations. The use of microbeads also makes it possible to compare the flow cytometry data over time, and between one laboratory and another.[34–36]

In order to investigate the clinical applicability and safety of GM-CSF therapy in patients with neoplasms of the myeloid compartment, and possibly to optimize its use in the clinical setting, we evaluated the cytofluorimetry expression of GM-CSF receptors in cells from patients with AML (n = 72), ALL (n = 18), MDS (n = 12), and from 12 healthy subjects. The number of GM-CSF receptors per cell was measured with a FacScan flow cytometer, using quantitative calibration microbeads which allowed a precise calculation of the number of GM-CSF/R per cell in the various populations analyzed.

Materials and methods

Subjects

Fresh bone marrow (BM) cells obtained from patients with acute myeloid leukemia (n = 72), and acute lymphoblastic leukemia (n = 18) were investigated within 4 h from their collection. Peripheral blood cells from 12 patients with myelodysplastic syndromes, were also included in this study.

Peripheral blood and bone marrow cells obtained from 12 healthy adult subjects (aged between 18 and 55 years; mean, 36 years) served as controls.

Diagnosis of leukemia was based on morphological, cytochemical, cytogenetics, molecular genetics, and immunological criteria. According to FAB (French–British–American) classification, AML patients were distributed as follows: M0, seven patients; M1, 14; M2, 20; M3, two; M4, eight; M5, 20; M6, one. Patients with acute leukemia had a blast percentage higher than 60% in the specimen analyzed (mean 76%).

According to FAB criteria, patients with MDS were distributed as follows: refractory anemia (RA) (n = 6), refractory anemia with excess of blasts (RAEB) (n = 4), RAEB in transformation (RAEB-t) (n = 2).

The diagnosis of byphenotypic leukemias or AML with minimal phenotypic deviation was made according to the proposals of the EGIL group.[37]

AML patients were treated with a DAT (daunorubicin, cytosine arabinoside, thioguanine)-based induction regimen, followed by consolidation chemotherapy. Patients with ALL of B lineage (pro-B subtype) were treated according to a standard protocol based on the sequential use of daunorubicin, vincristine, cytosine arabinoside, cyclophosphamide, prednisone and asparaginase.

Flow cytometry analysis

Whole blood samples and purified CD34$^+$ cells were analyzed with a FacScan flow cytometer (Becton Dickinson, San José, CA, USA) equipped with a 15 W argon-ion laser. The instrument was calibrated with FITC (isothyocyanate of fluorescein) and PE (R-phycoerythrin) beads provided by Becton Dickinson. Data were analyzed with appropriate negative (isotypic) and positive (blood cells from healthy adult subjects) controls using Lysis II and Paint-a gate research software. Twenty thousand cells were analyzed for each sample at a flow rate of approximately 300 particles per s.[38]

The blast cell population was identified using a combined approach based on a multicolor analysis and evaluation of light scattering properties of the cells (forward scatter and side scatter).[39]

Flow cytometry analysis of GM-CSF receptor on CD34$^+$ bone marrow cells obtained from healthy subjects has been performed using a multiparametric live-gate approach. In three cases, the cytofluorimetric analysis was also performed on CD34$^+$ cells which were purified using a positive selection technique (Dynabeads; Dynal, Oslo, Norway).[35,40]

Flow cytometry data were expressed as percentage of positivity (using the standard marker approach), as molecules of equivalent soluble fluorochrome (MESF), and as antibody binding capacity (ABC).

Based on the analysis of the minimum detection threshold of our flow cytometer, and on variation in ABC value for GM-CSF/R in control and pathological samples (evaluated within and between different specimens), we estimated that the lower limit of quantitation was 500 molecules/cell, which corresponded to 1.0×10^3, MESF/cell. Below this limit the enumeration of antigenic determinants was considered unreliable, and unreproducible. However the lower limit of detection was variable and ranged from 390 to 550 molecules/cell.

Immunophenotypic analysis

Immunophenotypic profile of leukemic cells was assessed with a FacScan flow cytometer using a large panel of fluorochome-conjugated McAbs, including CD13, CD33, CD15, MPO (MPO-7, CLB-MPO1), CD34 (HPCA2), CD11b, CD11c, CD65, CD41, CD61, glycophorin A, CD35, CD117, CD56, Thy-1 (CD90), HLA-DR, CD38,CD14, CD10, CD19, CD22, CD3, CD5, CD7, cyCD3, cyCD22, CD79a,TdT (Becton Dickinson, San Jose, CA, USA; Dakopatts, Glostrup, Denmark; Immunotech, Marseille, France; Serotec, UK; Caltag).

Experiments of single, double, and triple staining were employed in all cases.

Monoclonal antibodies to GM-CSF/R

The expression of GM-CSF/R on normal and leukemic cells was assessed by using three different unconjugated McAbs:

(1) HGM-CSFR (Immunex, Seattle, WA, USA), M5D12 (supplier: Dr JS Thompson, University of Kentucky, Lexington, KT, USA) and 4B5F5 (supplier: Dr Ashman, Adelaide, Australia). HGM-CSFR McAb is directed to the GM-CSF/R alpha chain, while the epitope recognized by M5D12 and 4B5F5 McAbs is still uncertain. M5D12 and 4B5F5 McAbs were provided by Dr RT Todd (Myeloid Panel Chairman; Ann Arbor, MI, USA) and were intended for analysis during the Fifth Workshop on Leukocyte Differentiation Antigens held in Boston, in November 1993.[30] M5D12 McAb (CD116) is now available from Pharmingen (San Diego, CA, USA).

Negative controls with isotype-matched nonrelevant MoAbs (mouse IgG1, IgG2a, IgM; Dakopatts, Silenus, Australia) were done in all experiments. All the MoAbs were utilized using an indirect immunofluorescence technique. As second step, we employed FITC-conjugated rabbit F(ab') Ig fragments specific for mouse Ig (fluorochrome/protein ratio: 2.3; Dakopatts).

Whole blood cells were washed twice in PBS before their incubation with GM-CSF/R McAb in order to remove serum GM-CSF. Human AB serum was added prior to antibody incubation to avoid nonspecific binding of McAbs to Fc receptors. All samples were further incubated for 10 min with a lysing buffer (Facslysing solution; Becton Dickinson) to eliminate red blood cells.

MESF and ABC calculation

Quantum beads: To evaluate antigen expression for the various GM-CSF/R McAbs, we used FITC quantitative microbead calibration standards (Quantum 26p beads; Flow Cytometry Standard Corporation, Milan, Italy, purchased from Walter Occhiena srl, Torino, Italy) as a reference standard to compare to the fluorescence intensity of cells stained for the various antibodies. The use of calibrated microbeads made it possible to express the flow cytometry data in the form of molecules of equivalent soluble fluorochrome (MESF) values. The calculation of MESF is based on the evaluation of the peak channel expressed by cells and different populations of microbeads. The value for the slope, intercept, and correlation coefficient allow the calculation of the regression line, which gives information on the linearity and stability of the instrument response, the noise level of the flow cytometer, the MESF values of the stained and unstained cells, the resolution indexes for fluorescence, and the coefficient of variation (CV) of microbeads fluorescence.[35] The minimum detection threshold of the instrument corresponds to the MESF value for the blank beads, and provides information on the sensitivity of the flow cytometer in detecting fluorescence signals, which resulted for our FacScan within 130–310. Based on the protein/fluorochrome ratio of the second step, the antibody binding capacity may be calculated for each cell population.

After data acquisition, the microbeads were run again, in order to see whether any significant changes in MESF values occurred. In that case, MESF calculation of the sample was corrected accordingly.

For each sample, the MESF value of the isotypic control was reduced from the MESF value of the positive cell population.

QIFIKIT: Starting from the beginning of 1995, normal and acute leukemic samples were also analyzed by using the DAKO QIFIKIT (Dakopatts). This kit contains a series of beads coated with different, but well defined quantities of mouse monoclonal antibody molecules. These beads mimic cells with different antigen densities which have been labeled with a primary mouse monoclonal antibody, isotype IgG, and they serve as a set of standards to calibrate the fluorescence scale of the flow cytometer in units of ABC.

CFU assays

The colony-forming unit granulocyte–macrophage (CFU-GM), CFU-GEMM, and BFU-E assays were performed by plating 1×10^5 light density (<1.068 g/ml) mononuclear cells/plate. Cells were cultured in 35-mm diameter standard tissue culture dishes (Flow) containing 0.9% methylcellulose (MethoCult H4230; Stem Cell Technologies, Vancouver, Canada), 30% pretested FBS, 1% pretested BSA, 10^{-4} M 2-mercaptoethanol, with 10% supernatant of the 5637 cell line added and 1 U/ml human urinary erythropoietin. The cells were cultured for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Colonies (>50 cells) were scored at days 7 and 14. All cultures were performed in triplicate.[41]

GM-CSF receptors modulation studies

Experiments of GM-CSF receptors modulation were carried out *in vitro* using a liquid culture assay. 1×10^5/ ml blasts from 10 patients with GM-CSF/R+ AML were seeded in 25-cm^2 tissue culture flasks (Falcon, Bedford, USA) containing RPMI culture medium, supplemented with FCS 10%, and 10 ng/ml of rhGM-CSF (Boehringer, Mannheim, Germany). Cultures were incubated at 37°C for 48 h in a humidified 5% CO_2 in air. Flow cytometry analysis of GM-CSF/R expression was performed at day 0, and 2 h and 24 h after GM-CSF treatment, by using HGM-CSFR McAb. Cells with no GM-CSF added to the medium were used as controls.

p53 protein expression analysis

Two monoclonal antibodies PAb 1801 and PAb 240 (Oncogene Science, Cambridge, UK), were used to detect p53 expression. The antibody PAb 1801 recognized an epitope near the NH2-terminal end of the p53 peptide present in all species studied; it reacts preferentially with human p53. The antibody PAb 240 was originally reported to recognize a common conformational epitope closely associated with undenatured wild-type p53 protein in hemopoietic cells.

For the detection of p53 expression by flow cytometry, cells were first fixed at −8°C to −10°C for 5 min in paraformaldheyde-lysine-periodate (PLP) fixation solution.[40,42] After removal from the fixation solution, cells were washed in phosphate-buffered saline and incubated with one or another of the p53 monoclonal antibodies for 30 min at 4°C, washed in phosphate-buffered saline, and incubated with fluorescein isothiocyanate-conjugated rabbit antimouse IgG monoclonal antibody (Dakopatts) before incubation with the conjugated secondary antibody. Flow cytometric analysis was carried out on a FacScan flow cytometer.

Assessment of DNA content by flow cytometry

The assessment of cellular DNA content was made with a FacScan flow cytometer (Becton Dickinson). The cell cycle distri-

bution data were provided by the Rectangle-Fit (R-FIT) mathematical algorithm of the FacScan/Cellfit software program. The double discrimination module was used to detect doublets and higher cell aggregates. To determine DNA content, cells were treated with 0.5% Triton X-100 for 3 min and then stained with a propidium iodide solution (50 g/ml). RNase (Sigma), at a final concentration of 0.1%, was added to the cell cycle preparation.[41]

Cell proliferation studies

Bromodeoxyuridine incorporation and a monoclonal antibody against bromodeoxyuridine (Becton Dickinson) in conjunction with flow cytometry were used to calculate the size of population of cells in S-phase of the cell cycle. For labeling cells and detection we used a previously published method.[41]

Chromosome analysis

Cytogenetic studies of leukemic cells were performed at leukemia diagnosis to detect the presence of structural or numerical abnormalities. It was based on both a direct technique or a short-term culture. Synchronization with methotrexate and thymidine was carried out. G-banding technique was applied, and karyotypes were expressed according to the International System for Human Cytogenetic Nomenclature.

Statistical analysis

GM-CSF receptor expression and clinical and biological characteristics were compared using parametric and nonparametric statistics (Student's *t*-test, Wilcoxon test, linear regression model, multivariate regression analysis). When pertinent, one-way analysis of variance (ANOVA) and χ^2 or Fisher tests were used to compare means and frequencies. Kaplan–Meier technique was used in estimating overall survival and relapse-free survival of GM-CSF/R+ve *vs* GM-CSF/R-ve AML patients.

Results

Analysis of GM-CSF-receptor in blood and bone marrow hemopoietic cells from healthy subjects

The flow cytometry data derived from the quantitative analysis of expression levels of GM-CSF receptors in peripheral blood and bone marrow cells from 12 healthy donors are shown in Table 1. The number of receptors per cell was four-fold higher in monocytes than in neutrophil granulocytes, whereas lymphocytes did not express detectable levels of GM-CSF receptors.

As far as the cytofluorimetric expression of GM-CSF/R in bone marrow cells is concerned, we found that myeloid precursors (promyelocytes, myelocytes, and metamyelocytes) showed intermediate levels of GM-CSF/R (range of MESF/cell: 11.7×10^3–40.5×10^3 ABC), as demonstrated by two- and three-color fluorescence analysis (CD33, HGM-CSFR, CD45). Monocytes and their precursors showed higher levels of GM-CSF/R (MESF range: 25.7×10^3–69×10^3), as assessed by multicolor fluorescence experiments (CD4, HGM-CSFR, CD45 or CD13).

In addition, on the basis of the quantitative analysis of expression levels of GM-CSF receptors, two different subsets of GM-CSF/R$^+$/CD34$^+$ cells were identified: GM-CSF/R^{dim} (showing a mean MESF/cell value of 2.5×10^3), and GM-CSF/R^{bright} (10% of the total number of CD34 cells: mean: 22.0×10^3 MESF/cell). Data derived from two- and three-color experiments indicated that the GM-CSF/R^{bright} cell fraction comprised both lineage (Li)$^+$ (mean percentage of positivity: 5.2 ± 3.1 s.d.) and Li$^-$ (HLA-DR$^-$, CD38$^-$, CD33$^-$) (mean %: 3.0 ± 2.2 s.d.) CD34$^+$ cells. A similar pattern was observed for the GM-CSFR^{dim} subset, which was detected in Li$^+$ (mean %: 49.6 ± 10 s.d.) and Li$^-$ (2.9 ± 1.9 s.d.) CD34$^+$ cells.

Furthermore, the expression levels of GM-CSF/R of normal hemopoietic cells did not show any positive correlation with the number of CFU-GM or CFU-GEMM colonies, or with active cell cycle phases.

Analysis of GM-CSF-receptor in MDS cells

The number of GM-CSF/R expressed by neutrophil granulocytes and monocytes from patients with MDS was significantly lower than that of their normal counterparts; this reduction was more prominent in patients with a bone marrow blast percentage higher than 10% of nucleated cells (Table 1). Furthermore, flow cytometry analysis showed that 80% of MDS patients presented a loss of receptors for GM-CSF in 6–73% of cells (mean 19%).

Analysis of GM-CSF receptor in blast cells from acute leukemia

The number of AML patients showing positivity for GM-CSF varied significantly from case to case, depending on the McAb used to detect GM-CSF/R, the FAB subtype, and/or the clinical phase of the disease. M5D12, 4B5F5, and HGM-CSFR McAbs were found to be positive in 33, 90, and 78%, respectively, of the cases examined (range of MESF/cell: 1.4×10^3–106.7×10^3). The highest expression was seen in patients classified as M5 FAB subvarieties (mean MESF: 39.4×10^3), while the lowest expression was found in the M0 FAB category (mean MESF: 3.0×10^3) (Table 2, Figure 1). Two- and three-color analysis further revealed that over 70% GM-CSF/R$^+$ blasts from patients belonging to the M1-M2 FAB subtype co-expressed CD34^{low}, c-kit/R$^+$, HLA-DR^{high}, CD33, CD38 antigens, and had little or no capacity of forming CFU-GM colonies. Within the M4 and M5 FAB subtypes, GM-CSF/R$^+$ blasts co-expressed CD11c, CD14, CD13^{low}, CD33, and CD87^{bright}.

In AML, no correlation was found between the number of GM-CSF/R and kinetic status, DNA content, p53 protein expression, and cytogenetic abnormalities (*P* and *r* values not significant; data not shown).

In AML, there was no difference in overall survival and complete remission rates between patients whose blasts cells expressed GM-CSF/R ($n = 56$) and those who resulted GM-CSF/R negative ($n = 16$) (data not shown).

Blast cells from all but two ALL patients were GM-CSF/R negative. However, the number of receptors detected in GM-CSF/R$^+$ ALL patients was extremely low (<1000 ABC/cell; <2300 MESF/cell). In the first case, we observed a co-expression of myeloid (CD13 and CD33) and lymphoid markers (CD10, CD19, CD79α, CD22). The remaining case was classified as pro-B ALL (CD79α^+, CD10$^+$, CD19$^+$, CD22$^+$).

The quantitative analysis of GM-CSF/R in acute leukemia

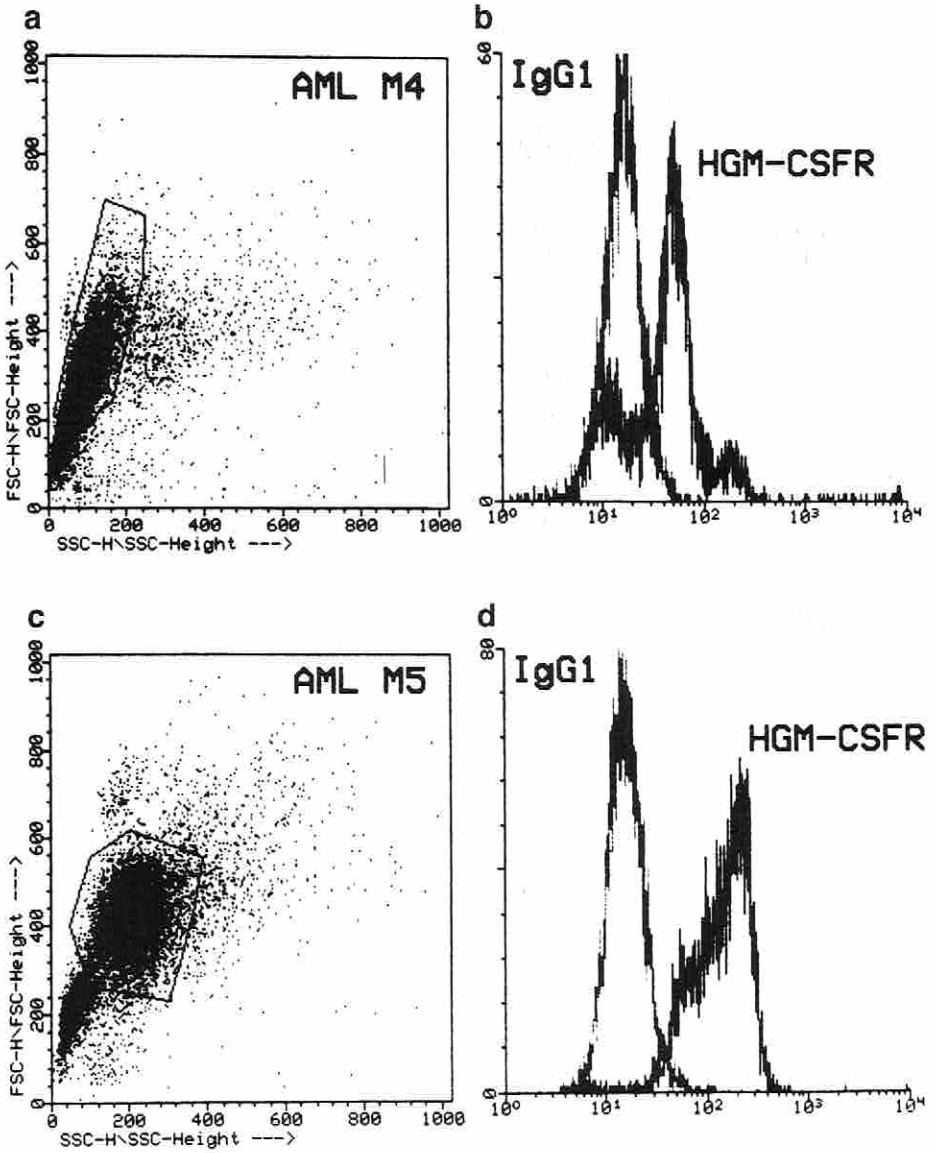

Figure 1 Quantitative analysis by flow cytometry of GM-CSF receptors on acute leukemic blasts from a patient with AML FAB M4 (a, b) and FAB M5 (c, d). The light scattering properties of the cells from the same two patients are shown in the left panels (a, c). IgG1, Histogram distribution of the isotypic control; H-GMCSFR, anti-GM-CSF/R McAb.

further showed that the number of receptors expressed by five out 72 (7%) AML cases was above the highest values seen in normal samples (>69.2×10^3 MESF/cell; range, 77.3×10^3–106.7×10^3). All these cases were found within the M5 FAB subtype, where receptor overexpression was noticed in 25% of the cases examined (Figure 2).

Regarding the reactivity of the three GM-CSF/R McAbs used in this study, a positive correlation between expression of HGM-CSFR and M5D12 McAbs in both normal and leukemic cells was found, while the pattern of reactivity of HGM-CSFR and 4B5F5 McAb, and M5D12 and 4B5F5 McAb was significantly different (r values, not significant).

The statistical analysis of 22 samples showed that both the indirect immunofluorescence staining in the Quantum beads assay, and that in the QIFIKIT assay give comparable quantitative data. A positive correlation was noticed between both MESF and ABC values in the Quantum assay, and ABC in the QIFIKIT ($r = 0.992$, and $r = 0.99$, respectively) (Figure 3).

Interestingly, in three AML patients, relapsed disease after induction–consolidation chemotherapy was characterized by a two-fold increase in GM-CSF receptor number (mean pre-treatment ABC values: 3.5×10^3/cell; mean ABC value in relapsed disease: 7.3×10^3/cell). In the same cases, we observed a marked increase in the number of proliferating cells, ie S-G2+M cell cycle phases (pretreatment values: 5.1%; relapse: 14.2%).

GM-CSF receptor modulation studies in AML

Cell culture studies aimed at evaluating GM-CSF receptor modulation following AML blast exposure to GM-CSF showed two distinct patterns of response: in the first group of patients ($n = 6$), GM-CSF down-modulated GM-CSF receptors, while in the second group, the administration of GM-CSF was associated with either an increase ($n = 2$) or no change ($n = 2$) in the number of GM-CSF/R (Figure 4). In brief, when incubated in the pres-

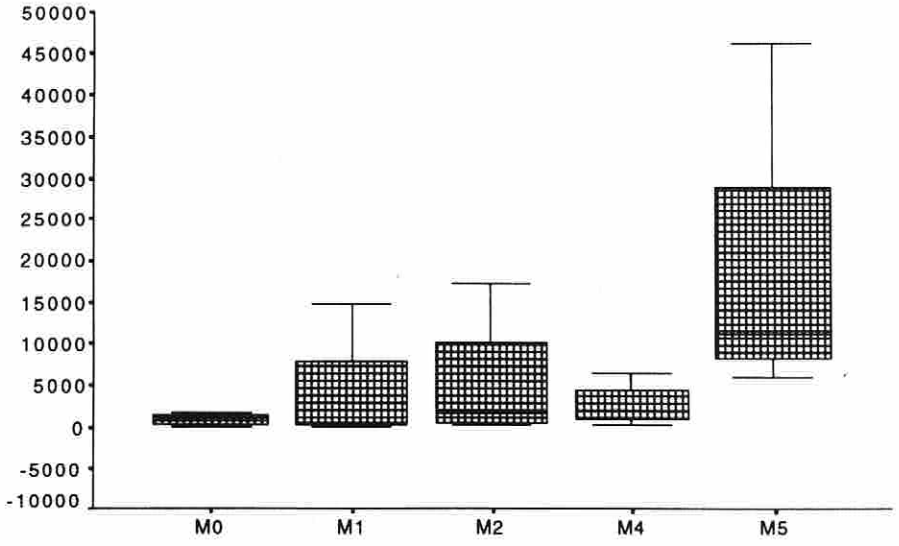

Figure 2 Expression levels of GM-CSF receptors in AML blasts according to the FAB subtype (box plot representation). Data were obtained using the HGM-CSFR McAb (CD116). Acute leukemic blasts from five AML patients with M5 FAB subtype had a number of GM-CSF/R significantly higher than that of normal bone marrow hemopoietic cells. Vertical axis, ABC value.

Table 1 Flow cytometry quantification of GM-CSF/R in normal and MDS peripheral blood cells using HGM-CSFR McAb and Quantum beads

Cell population	*GM-CSF/R expression in PB and BM hemopoietic cells from healthy adults* *% positive cells*[a] *MESF/cell x 10^3: mean (range)*[b] *ABC/cell x 10^3: mean (range)*[b]	*P value*	*GM-CSF/R expression in PB and BM cells from MDS* *% positive cells* *MESF/cell x 10^3: mean (range)*[b] *ABC/cell x 10^3: mean (range)*[b]
PB neutrophil granulocytes	97.1 7.4 (3.2–14.1) 3.23 (1.4–6.1)	<0.01 <0.001 <0.001 NS <0.001	78.3 3.7 (1.1–9.6) 1.6 (0.5–4.2) RA (ABC): 2.2 (1.1–4.2) RAEB (ABC): 1.0 (0.5–3.0)
PB monocytes	98.2 36.1 (17.0–60.2) 15.7 (7.4–26.2)	<0.01 <0.001 <0.001 <0.05 <0.001	67 21.6 (7.1–38.8) 9.4 (3.1–16.9) RA (ABC): 12.7 (6.8–16.9) RAEB (ABC): 6.8 (3.1–13.0)
BM myeloid precursors	95.3 25.5 (11.7–40.5) 11.1 (5.1–17.6)	<0.01 <0.05 <0.05	64 15.4 (7.6–31.5) 6.7 (3.3–13.7)
BM monocytic precursors	97.5 41.9 (25.7–69) 18.2 (11.2–30.0)	<0.05 NS NS	70.2 30.8 (10.3–58.6) 13.4 (4.5–25.5)
BM CD34+ cells GM-CSF/R^{dim}	61.1 2.5 (0.9–4.1) 1.1 (0.4–1.8)		ND
BM CD34+ cells GM-CSF/R^{bright}	7.4 22.1 (11.5–34.0) 9.6 (5.0–14.8)		ND

[a]Data are expressed as a mean of positive cases.
[b]The range for ABC and MESF values refers to positive cases.
BM, bone marrow; PB, peripheral blood; ND, = not done; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess of blasts; NS, not significant.

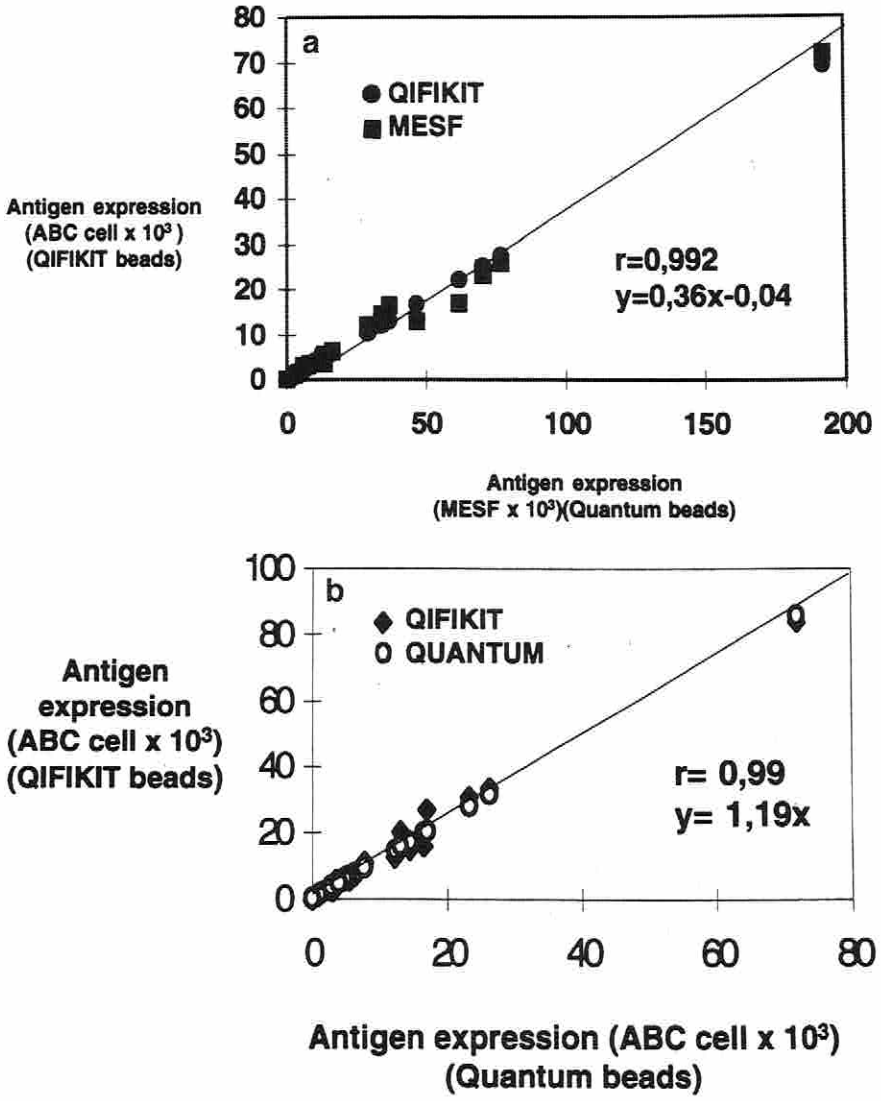

Figure 3 Comparative cytofluorimetric analysis of GM-CSF-R with the Dako QIFIKIT test and the Quantum assay. Panel a shows the comparison between ABC values expressed by normal and leukemic cells in the QIFIKIT assay (y axis) and MESF values in the Quantum assay (x axis). The correlation between ABC values in the QIFIKIT test (y axis) and ABC values seen in the Quantum beads assay (x axis) is shown in (b). ABC calculation in the Quantum test was based on the use of a second step (FITC-conjugated rabbit F(ab') Ig fragments) of 2.3 fluorochrome/protein ratio. Data were obtained using the reference HGM-CSFR McAb. The comparative analysis was performed on 23 samples (five normal and 18 leukemic).

ence of rhGM-CSF, leukemic cells from six AML patients showed a 71% down-regulation of GM-CSF/R; this decrease was statistically significant ($P < 0.001$). In contrast, no significant changes were observed in cell cultures obtained from leukemic cells incubated without rhGM-CSF. In two cases, a 37% up-regulation of GM-CSF/R was observed after priming AML cells with rhGM-CSF.

Discussion

In this study, a quantitative cytofluorimetric analysis of the expression levels of GM-CSF/R was carried out on patients affected by various hematological malignancies (AML, ALL, MDS), and on 12 healthy adult volunteers, with the aim of providing a better selection of patients with clonal myeloid disorders who may be eligible for GM-CSF treatment.[43,44] Moreover, the biologic and clinical significance of GM-CSF receptor expression in acute leukemia patients was investigated.[45–50] To detect the receptor for GM-CSF, three different unconjugated GM-CSF/R McAbs were used (HGM-CSFR, M5D12, 4B5F5) in conjunction with a flow cytometer and appropriate standards. Flow cytometry data were expressed in the form of both MESF and ABC/cell through the use of FITC-pre-mixed quantitative microbead calibration standards, and QIFIKIT calibration beads.[34–35]

In healthy subjects, reactivity for HGM-CSF/R McAb was detected on peripheral blood monocytes, neutrophils, and bone marrow (BM) myelo-monocytic precursors. In addition, two distinct subsets of normal BM $CD34^+$ progenitors showed positivity for HGM-CSFR McAb: GM-CSF/R^{dim}, and GM-CSF/R^{bright} (10% of the total number of CD34 cells). GM-CSF/R^{bright} cell fraction was found within both Li^+ and Li^- $CD34^+$ cells; similarly, a GM-$CSFR^{dim}$ subset was identified in Li^+ and Li^- $CD34^+$ cells. Furthermore, in healthy donors there was no correlation between the number of GM-CSF/R detected by flow cytometry, CFU-GM/CFU-GEMM colony production, or cell cycle phases. These

Table 2 Flow cytometry quantification of expression levels of GM-CSF/R in acute leukemic blasts of myeloid (AML), B lymphoid (B-ALL) and mixed lineage (BAL) (data obtained using Quantum beads)

AML FAB subtype	*HGM-CSFR McAb*	*4B5F5 McAb*	*M5D12 McAb*
	% positive cases[a] *MESF/cell x 10^3: mean (range)*[b] *ABC/cell x 10^3: mean (range)*[b]	*% positive cases* *MESF/cell x 10^3: mean (range)*[b] *ABC/cell x 10^3: mean (range)*[b]	*% positive cases* *MESF/cell x 10^3: mean (range)*[b] *ABC/cell x 10^3: mean (range)*[b]
AML M0[c] ($n = 7$)	66 3.0 (2.8–3.4) 1.3 (1.2–1.5)	71 39.1 (27.8–55.4) 17.0 (12.1–24.1)	14 1.8 (3.2–5.3) 0.8 (1.4–2.3)
M1 ($n = 14$)	64 14.0 (1.1–33.5) 6.1 (0.5–14.6)	93 26.9 (6.0–35.6) 11.7 (2.6–15.5)	21 4.8 (3.4–6.2) 2.1 (1.5–2.7)
M2 ($n = 20$)	65 13.1 (1.1–39.3) 5.7 (0.5–17.1)	90 23.2 (5.1–64.6) 10.1 (2.2–28.1)	25 21.6 (0.9–46) 9.4 (0.4–20.0)
M3 ($n = 2$)[d]	100 18.6 (6.4–30.8) 8.1 (2.8–13.4)	100 17.2 (8.0–26.4) 7.5 (3.5–11.5)	100 2.5 (1.6–3.5) 1.1 (0.7–1.5)
M4 ($n = 8$)	88 15.2 (1.6–27.1) 6.6 (0.7–11.8)	87 22.5 (10.3–29.2) 9.8 (4.5–12.7)	37 2.1 (1.1–3.2) 0.9 (0.5–1.4)
M5 ($n = 20$)	100 47.8 (18.4–106.7) 18.2 (8.0–46.4)	95 46.7 (24.1–111.3) 20.3 (10.5–48.4)	50 24.4 (2.7–70.4) 10.6 (1.2–30.6)
M6 ($n = 1$)[e]	100 46.7 20.3	100 60.7 26.4	0
B-lineage ALL ($n = 18$)	11 1.1 (1.0–1.4) 0.5 (0.44–0.6)	11 2.8 (1.6–3.9) 1.2 (0.7–1.7)	11 1.4 (1.4–1.5) 0.63 (0.60–0.66)
BAL ($n = 3$)	66 2.5 (1.8–3.2) 1.1 (0.8–1.4)	33 3.2 1.4	33 2.7 1.2

[a]Data are expressed as mean percentage of positive cases.
[b]Ranges for MESF and ABC refer to positive cases.
[c]FAB (French–American–British) subtype.
[d]Cell positivity was evaluated on leukemic promyelocytes.
[e]GM-CSF/R expression was detectable on both erythroid (CD71$^+$, glycophorin-A negative) and myeloid blasts.
BAL, byphenotypic acute leukemia.

data indeed support the concept that GM-CSF receptors are expressed on myeloid cells throughout their maturational pathway,[45–49] as well as on subsets of bone marrow progenitors.

As far as the expression of GM-CSF/R in MDS cells is concerned, we found that the number of GM-CSF/R expressed by neutrophil granulocytes and, to a lesser extent, by monocytes from MDS patients was significantly lower than that from their normal counterparts; this reduction was more prominent in patients with a bone marrow blast percentage over 10% of the nucleated cells. Furthermore, the flow cytometry analysis showed that 80% of MDS patients presented a loss of GM-CSF receptors in 6–73% of cells.

Among AML samples, M5D12 McAb was positive in 33%, 4B5F5 McAb in 90%, and H-GMCSF/R McAb in 78% of the cases examined. However, whereas 58% of patients from M0-M1 and M2 FAB subtypes expressed GM-CSF/R in over 40% of leukemic cells, all patients with M5 FAB subtypes showed positivity for HGM-CSF/R McAb in the majority (>80%) of blast cells. Furthermore, blasts from FAB M5 AML were characterized by the highest receptor numbers, thus supporting the concept that a close association between the monocytic commitment and expression of GM-CSF/R may exist. In most cases of M1-M2 FAB subtypes, GM-CSF/R$^+$ blasts co-expressed CD34^{low}, c-kit/R$^+$, HLA-DR^{high}, CD33, CD38 antigens, and had little or no capacity of forming CFU-GM colonies. Within M4 and M5 FAB subtypes, GM-CSF/R$^+$ blasts co-expressed CD11c, CD14, CD13^{low}, CD33, CD87^{bright}. In AML, GM-CSF/R expression did not correlate with kinetic status, DNA content, p53 protein expression, or cytogenetic abnormalities.

On the contrary, only 2/18 cases of B lineage ALL showed a dim GM-CSF/R positivity. Recent reports have shown that GM-CSF receptors can be found on some normal and leukemic lymphocytes, allowing the authors to speculate that receptor expression is a stage-specific feature of late B cells.[48] There is evidence that GM-CSF receptors are sometimes detectable in acute leukemic blasts of lymphoid lineage, thus supporting the concept that a small number of non-functional GM-CSF receptors may be detected in the early phases of lymphocyte commitment.[30]

Furthermore, the quantitative analysis of GM-CSF/R in acute leukemia has shown that the number of receptors expressed by five out of 72 (7%) AML cases was above the highest values seen in normal samples, allowing the possibility of using this marker for the monitoring of the minimal residual disease (MRD)

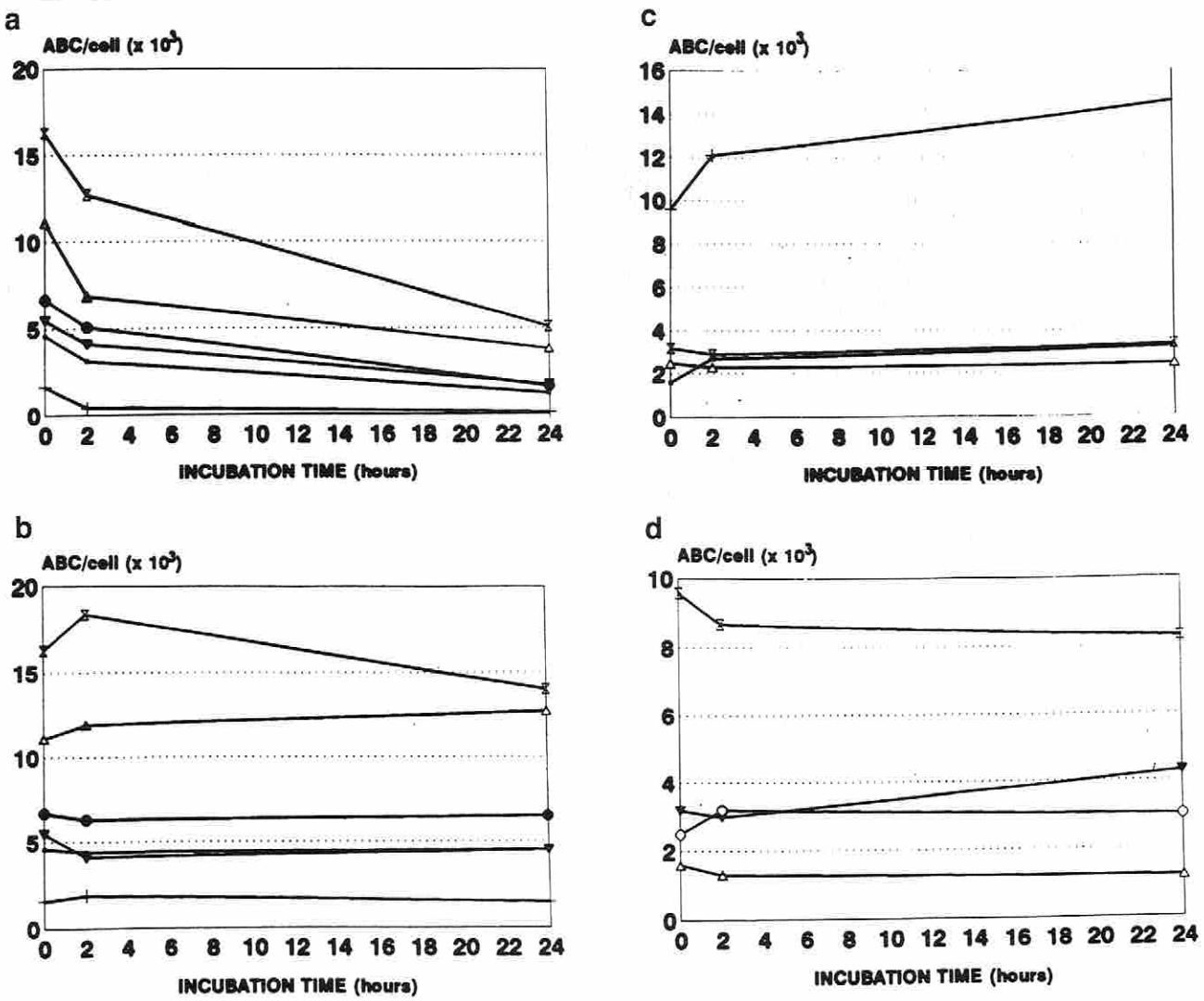

Figure 4 Effects of rhGM-CSF on GM-CSF receptor expression of AML cells. Flow cytometry analysis of GM-CSF/R was performed before and after *in vitro* cell incubation for 24 h at 37°C in the absence or presence of rhGM-CSF. The cytofluorimetric enumeration of GM-CSF/R was performed at time 0, and after 2 and 24-h incubation. Data are expressed in the form of ABC value. (a) Effects of rhGM-CSF on GM-CSF/R down-regulation ($n = 6$ patients); (b) fluorescence profiles of blast cells from the same AML patients shown in (a) incubated in the absence of rhGM-CSF; (c) fluorescence profiles of blast cells from four AML patients incubated with rhGM-CSF. In two cases, an up-regulation of GM-CSF/R was observed, while in the remaining two cases no change in receptor numbers was detected by flow cytometry. (d) Fluorescence profiles of blast cells from the same AML patients shown in (c) incubated in the absence of rhGM-CSF.

in a subset of AML. Another interesting finding which may have clinical implications is represented by the marked increase in the number of GM-CSF receptors in relapsed disease after induction–consolidation chemotherapy. In our cases, this finding was associated with a significant increase in the blast cell proliferation rate, thus suggesting that the quantitative analysis of this receptor may be valuable for the monitoring of AML patients.

Concerning the clinical implication of GM-CSF receptor studies in AML patients, it is likely that new insights into the use of rhGM-CSF in myeloid malignancies may derive from *in vivo* and *in vitro* studies aimed at evaluating GM-CSF receptor modulation, following exposure of cells to GM-CSF.[51–58] Some recent papers have shown that the affinity status and/or number of receptors can be significantly modified after treatment with GM-CSF.[51,53] There is also evidence that AML and MDS blasts not only express GM-CSF receptors but also synthesize GM-CSF themselves; as a consequence, they can stimulate their own proliferation (autocrine effect).[2,3,58] This effect can be interrupted by the administration of exogenous growth factors.[58]

In this paper, we have demonstrated that the exposure of GM-CSF/R$^+$ AML blasts to rhGM-CSF induced a 71% down-regulation of GM-CSF receptors in 60% of the cases examined, while in the remaining cases the administration of rhGM-CSF was associated with either an increase (37% up-regulation) or no change in the number of GM-CSF/R. These data may have clinical and therapeutic relevance, as GM-CSF is currently being used in patients with acute leukemia to shorten the duration of neutropenia following chemotherapy, or to recruit blast cells into active cell cycle phases when administered before chemotherapy. We personally believe that the dynamic evaluation of the receptor status on the blastic and 'supposed' normal cell population represents a promising tool for optimizing GM-CSF treatment in patients with acute leukemias, making the analysis of data from clinical trials based on the combined use of chemotherapy and GM-CSF more accurate and meaningful.

In theory, a careful evaluation of the cellular regulation of GM-CSF receptors after exposure of cells to rhGM-CSF could also help distinguish AML patients suitable for GM-CSF treatment to stimulate neutrophil recovery from those eligible for a chemotherapy- and GM-CSF-based strategy to recruit blast cells into the cell cycle. We propose that the first group should include patients whose leukemic cells contain low levels of GM-CSF receptors which are downmodulated by rhGM-CSF, while the second group should include subjects in whom the pathological clone is characterized by high–intermediate levels of GM-CSF receptors which are slightly and transiently down-regulated after ligand binding. In addition, it is likely that AML patients at high risk of infection-related mortality (including elderly and relapsed patients, or those resistant to induction–consolidation chemotherapy) whose leukemic cells express low–intermediate GM-CSF receptors may benefit from rhGM-CSF therapy in order to stimulate the recovery of neutropenia and/or monocytopenia after chemotherapy.[3,9]

In conclusion, cellular GM-CSF/R expression was variable and ranged from undetectable (ALL and a minority of AML) to very high intensities in M5 AML, and were also documented in more than 50% of M0 AML, thus supporting the concept that GM-CSF/R may be considered a sensitive and specific marker to detect leukemic cells committed to the myeloid lineage. The measurement of GM-CSF receptors may also offer the possibility of optimizing the *in vivo* use of rhGM-CSF in patients with neoplasms of the myeloid compartment, possibly improving its applicability and safety in the clinical setting.[59,60] However, a clinical study aimed at evaluating the clinical implications of a dynamic analysis of the density and affinity status of GM-CSF/R following *in vivo* exposure of cells to GM-CSF in AML patients may lead to a better understanding of the rationale of applying rhGM-CSF in the treatment of AML. Hopefully, this approach should reduce the risk of stimulating the proliferation of leukemic cells following intensive myeloablative chemotherapy in at least one subset of AML.

Acknowledgements

This work was supported by grants from MPI 40%, Regional Funds, AIRC (Associazione Italiana per la Ricerca sul Cancro), CNR (ACRO project), and Progetto Sangue (Istituto Superiore Sanità). We are grateful to Dr Armitage, Dr Elaine K Thomas, Dr Michael B Widmer (Immunex, Seattle, USA) for providing us the HGM-CSF-R McAb, and to Prof RT Todd (Ann Arbor, USA) for giving us M5D12 and 4B5F5 McAbs.

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