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Growth advantage of chronic myeloid leukemia CFU-GM *in vitro*: survival to growth factor deprivation, possibly related to autocrine stimulation, is a more common feature than hypersensitivity to GM-CSF/IL3 and is efficiently counteracted by retinoids ± **α**-interferon

D Ferrero, C Foli, F Giaretta, C Argentino, C Rus and A Pileri

Divisione di Ematologia dell'Università di Torino, Azienda Ospedaliera S Giovanni Battista, Torino, Italy

Bcr/abl fusion gene, in experimental models, induces survival to growth factor deprivation and hypersensitivity to IL3. However, conflicting data were reported about chronic myeloid leukemia (CML) progenitors. We investigated the responsiveness of purified CML CFU-GM to GM-CSF/IL3 and their survival to growth factor deprivation. CFU-GM hypersensitivity to IL3 and/or GM-CSF was found in 3/11 CML cases only. CML CFU-GM survived well in stroma-free 'mass' culture (5×10^4 cells/ml) without cytokine addition, up to day 11, average recovery being around 95% in medium + 10% fetal bovine serum and 67–81% in serum-free medium. Conversely, normal progenitors declined steadily, particularly after extensive purification (18 ± 10% recovery at the 7th day), and in serum-free medium (4 ± 6% recovery). By contrast, normal and CML CFU-GM declined in a similar way in limiting dilution cultures (1–10 cells/50 μl). We also investigated the effects of retinoic acid and α-interferon on CFU-GM survival. Both all-*trans*- and 13-*cis* retinoic acid, particularly in combination with α-interferon, reduced CML CFU-GM recovery down to normal progenitors' values. In conclusion, hypersensitivity to CSFs is rare in CML, whereas resistance to growth factor deprivation has been confirmed in mass, but not in limiting, dilution cultures. Both stereoisomers of retinoic acid, at therapeutic concentrations and in combination with α-interferon, can overcome the survival advantage of CML progenitors. *Leukemia* (2001) **15,** 422–429.
Keywords: CML CFU-GM; growth factor-deprivation; retinoic acid; α-interferon; GM-CSF; IL3

Introduction

The reciprocal translocation between bcr and cAbl genes, that characterises Ph[1] chromosome (t(9;22)(q34;q11)) of chronic myeloid leukemia (CML) cells, results in a chimeric gene,[1–4] whose product (bcr/abl fusion protein) induces several biochemical and functional changes in human and murine hematopoietic cells.[5–10] Survival and growth advantage are the final, more outstanding biological modifications resulting from the enhanced enzymatic activity of bcr/abl kinase; in particular, the transfection of a bcr/abl chimeric gene into interleukin 3 (IL3)-dependent murine myeloid cell lines induces resistance to the apoptotic stimulus exerted by growth factor deprivation.[11–15] Moreover, depending on gene expression level, hypersensitivity to or total independence from IL3 for *in vitro* growth, is also acquired.[15,16] Indeed, bcr/abl construct induces biochemical modifications, in particular tyrosine phosphorylation of several substrates, that mimic the effects of exogenous IL3 and GM-CSF.[17–19] Transduced bcr/abl chimeric gene was also reported to abrogate IL3/GM-CSF dependence of a human leukemic megakaryocytic cell line.[16] In that case, autocrine GM-CSF and IL3 production was induced by bcr/abl construct.[16] Excess production of IL3 and GM-CSF was also observed in a mouse model of myeloproliferative disease induced by bcr/abl transfection.[20]

In contrast to results obtained in experimental models, the biological mechanisms responsible for *in vivo* growth advantage of human CML progenitor/stem cells are not fully understood yet. *In vitro* resistance of late and early CML progenitor cells to some negative regulators of myelopoiesis, such as prostaglandins E[21] and MCP-1[22,23] was described. Autocrine G-CSF production by differentiating granulopoietic precursors in some CML cases[24] and increased M-CSF plasma levels[25] in most patients were also reported. More recently, a fraction of $CD34^+$ CML progenitor cells was found to be capable of autonomous, exogenous growth factor independent, proliferation *in vitro*. These cells were found to auto-produce IL3 and G-CSF.[26]

Conversely, somewhat conflicting results about growth factor requirement by CML progenitor cells were reported. Indeed, CFU-GM resistance to apoptosis induced by growth factor deprivation was evidenced in some,[14,27] but not in other studies.[28,29] Moreover, hypersensitivity to myeloid growth factors was not detected in typical, bcr/abl + CML,[14,30] whereas hyper-responsiveness to GM-CSF characterizes bcr/abl negative, juvenile CML.[30]

In this study, we compared the sensitivity to IL3/GM-CSF and the resistance to growth factor deprivation of highly enriched normal and CML myeloid progenitors. CFU-GM hypersensitivity to CSFs was detected in few CML cases only, whereas we could confirm the capability to survive growth factor deprivation as a quite common feature of leukemic progenitors, possibly related to autocrine growth factor production. Survival advantage of CML CFU-GM *in vitro* was efficiently counteracted by exposure to either all-trans- or 13-*cis* retinoic acid, particularly whether combined to α-interferon.

Materials and methods

Cell sources

Cells from both CML and control patients were collected during diagnostic procedures, after informed consent had been obtained.

Peripheral blood (PB) or bone marrow (BM) cells from CML patients in chronic phase were harvested either at diagnosis or during progressive leukocytosis, with WBC counts in excess of 25×10^9/l. All patients had recently undergone a karyotype analysis showing 100% Ph^1 + mitosis in their BM. In three cases, cells for karyotypic study were taken from the same samples of PB used for experiments. Control cells were from BM of patients not exposed to previous chemotherapy and not affected by diseases involving the myeloid lineage. In two

Correspondence: D Ferrero, Divisione di Ematologia dell' Università di Torino, Azienda Ospedaliera S. Giovanni Battista di Torino, via Genova 3, 10126 Torino, Italy; Fax: 39 011 6963737
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experiments, control cells were taken from leukapheresis collections performed for hematopoietic progenitor autotransplantation in non-Hodgkin's lymphoma patients.

Myeloid progenitor enrichment

Both BM and PB cells underwent the following three steps of progenitor cell enrichment: (1) density gradient separation on Ficoll-metrizoate (Lymphoprep; Nycomed, Oslo, Norway) at 1077 g/l; (2) phagocytosis of serum opsonized, heat inactivated yeast, followed by a second separation on Lymphoprep density gradient to remove mature myelo-monocytic cells;[31] (3) incubation with CD2 (Becton Dickinson, San Jose, CA, USA), CD11b (Becton Dickinson), CD19 (Coulter, Hialeah, FL, USA), anti-glycophorin (Dako, Glostrup, Denmark) and CD9 (supernatant of S17–12 hybridoma)[32] monoclonal antibodies, followed by immunomagnetic depletion with Dynabeads (Dynal, Oslo, Norway). The final proportion of $CD34^+$ cells, determined by direct immunofluorescence with a phycoerythrin-conjugated CD34 monoclonal antibody (Becton Dickinson) and cytofluorimetric analysis,[33] ranged between 10 and 60% (median 38%). In further experiments, highly purified progenitors (70–99% $CD34^+$ cells, median 87%) were obtained by substituting positive $CD34^+$ cell separation, through a Miltenyi Mini Macs magnetic cell separation system, (Miltenyi Biotec, Bergisch-Gladbach, Germany)[34] for the negative immunoselection.

CFU-GM assay

Hematopoietic progenitor-enriched cells were cultured at different concentrations ($1–5 \times 10^3$/ml, depending on the proportion of $CD34^+$ cells) in Iscove's modified Dulbecco's medium (IMDM) (GIBCO-Life Technologies, Paisley, UK) containing 20% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 0.3% agar (Difco, Detroit, MI, USA) and variable concentrations of either human recombinant GM-CSF (Myelogen, from Schering-Plough, Milan, Italy) or human recombinant IL3 (gift from Sandoz, Basel, Switzerland). Negative and positive control cultures were also established, with recombinant growth factors replaced by IMDM and 10% supernatant of 5637 cell line,[35] respectively. Colonies with at least 50 cells were scored after 14 days of culture.

Supernatant of 5637 bladder carcinoma cell line, containing a mixture of GM-, G-CSF and IL1,[35,36] was obtained from confluent cells (kindly provided by Dr G Rovera, The Wistar Institute, Philadelphia, PA, USA) cultured in IMDM + 10% FBS.

Liquid cultures

Cultures were established with progenitor-enriched cells, seeded at 5×10^4/ml in IMDM + 10% FBS with/without addition of either all-*trans* retinoic acid (ATRA) (from Sigma, St Louis, MO, USA) at 5×10^{-7} M or 13-*cis* retinoic acid (cisRA) (from Sigma) at 5×10^{-7}M or α-interferon (αIFN) (Wellferon; from Wellcome, London, UK) at 300 U/ml or a combination of ATRA + αIFN or *cis*RA + αIFN at the same concentrations reported above. Duplicate cultures were established for each condition and no sources of CSFs were added. In three CML and four control cases, highly enriched $CD34^+$ cells were also cultured in serum-free medium, containing IMDM + 10 μg/ml iron-saturated human transferrin (from Sigma) + 10 μg/ml human recombinant insulin (Actrapid, from Novo Nordisk, Bagsvaerd, Denmark) + 1% bovine serum albumin fraction V (from Sigma) + 4% lipid solution (from Sigma).

CFU-GM concentration in liquid cultures was evaluated at day 0 and after 4, 7 and 11 days: 50–100 μl from each cell suspension were harvested, washed with 6 ml of IMDM + 2% FBS and re-plated, for colony assay, in agar medium containing 10% supernatant of 5637 cell line, as above described. CFU-GM survival was evaluated by comparing their concentration in cell suspensions at day 0 and after 4, 7, 11 days of culture and expressed as mean percentage +/– s.d. of day 0 value.

Limiting dilution cultures

Highly purified $CD34^+$ cells from three CML and four control cases were cultured at 1–10 cells/round bottom microwell of 96 microwell plates in 50 μl of either IMDM + 10% FBS or serum-free medium. After 4 or 7 days, 50 μl of IMDM + 40% FBS + 20% 5637 SN were added to each well, to obtain final concentrations of 20–25% FBS and 10% 5637 SN. Fourteen days later, microwells were scored for clone growth (presence of at least 50 viable cells/well) and CFU-GM frequency was calculated by Poisson's statistics on the basis of the proportion of growth-negative wells at each cell concentration.[37] CFU-GM frequency in plates with late growth factor addition was compared to that detected in a control plate where cells were stimulated from day 0 with 20% FBS and 10% 5637 SN. This allowed the evaluation of CFU-GM proportion surviving the 4–7 days of growth factor deprivation.

Statistic analysis

The Wilcoxon Mann—Whitney test was used to analyze differences in CFU-GM recovery between normal and CML samples in control cultures. The same test was used to evaluate the differences in progenitor cell recovery determined by retinoids and/or αIFN.

Results

CFU-GM sensitivity to GM-CSF and IL3

CFU-GM responsiveness to both GM-CSF and IL3 was evaluated on seven control (BM) and 10 CML (three BM, seven PB) samples; the same cell sample was used in each case to test both growth factors. In one more CML case (PB) GM-CSF only was tested; in two more control (BM) and one more CML (PB) case, responsiveness to IL3 only was evaluated. $CD34^+$ cells represented a median 45% of plated cells (range 5–94) in CML cases and 23% (range 7–86) in controls.

Normal CFU-GM displayed maximal response to GM-CSF at concentrations ranging between 1 and 10 ng/ml, while 50% of maximal stimulation was achieved at 0.02–0.16 ng/ml (Figure 1). CFU-GM from most of the CML cases also displayed dose/response curves to GM-CSF that fitted in the above reported ranges; however, in 3/11 cases, a clearly higher sensitivity to the lowest GM-CSF concentration tested (0.02 ng/ml) was evident, with 80–90% of the maximal response (normal value 35 ± 21%) (Figure 1).

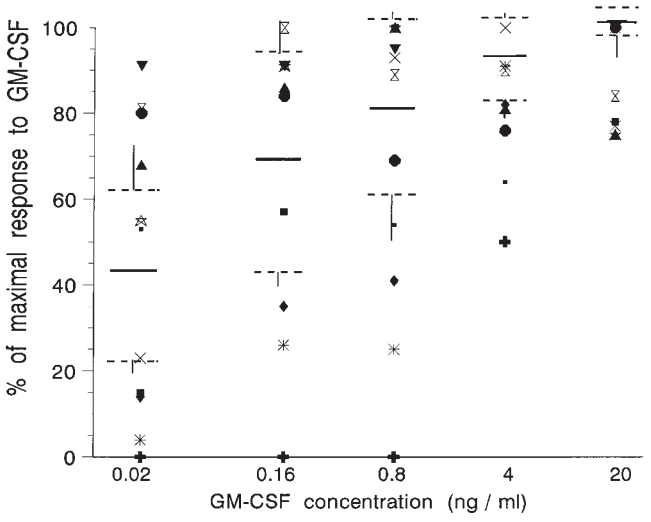

Figure 1 Responsiveness of normal and CML CFU-GM to increasing GM-CSF concentrations. Hematopoietic progenitor-enriched cells were cultured in agar medium with increasing GM-CSF concentrations for CFU-GM assay, as described in Materials and methods. CFU-GM growth is expressed as percentage of the maximum colony number obtained in the presence of GM-CSF as a stimulant (42–98 colonies/1–5×10^3 normal cells; 14–213 colonies/1–5×10^3 CML cells). Normal CFU-GM growth at each GM-CSF concentration is shown as mean value of seven cases (thick horizontal segment) ± s.d. (dashed horizontal segments) and range (vertical bars). The growth of CFU-GM from 11 CML cases, at each CSF consentration, is illustrated as symbols, each symbol referring to a different CML case.

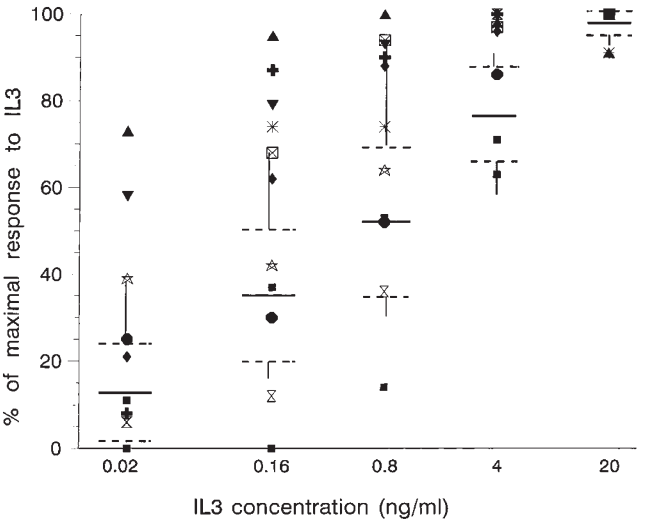

Figure 2 Responsiveness of normal and CML CFU-GM to increasing IL3 concentrations. Hematopoietic progenitor-enriched cells were cultured in agar medium with increasing IL3 concentrations for CFU-GM assay, as described in Materials and methods. CFU-GM growth is expressed as percentage of the maximum colony number obtained in each case in the presence of IL3 as a stimulant (16–102 colonies/1–5×10^3 normal cells and 14–125 colonies/1–5×10^3 CML cells). Normal CFU-GM growth at each IL3 concentration is shown as mean value of eight cases (thick horizontal segment) ± s.d. (dashed horizontal segments) and range (vertical bars). The growth of CFU-GM from 12 CML cases, at each IL3 concentration, is illustrated as symbols, each symbol referring to a different CML case.

Maximal and 50% response to IL3 by normal CFU-GM were reached at concentrations of 4–20 ng/ml and 0.16–2 ng/ml, respectively (Figure 2). CFU-GM from most of the CML cases evidenced normal dose/response curves in the presence of IL3. However, a definitely higher CFU-GM sensitivity to that cytokine was observed in two of the three cases who also displayed hypersensitivity to GM-CSF, reaching 55% and 75%, respectively, of the maximal response at the minimal (0.02 ng/ml) IL3 concentration tested (normal value 15 ± 12%) (Figure 2).

Two cases displayed a subnormal response to GM-CSF (50% stimulation at 2–4 ng/ml, Figure 1); one of these responded poorly to IL3 too (no colony growth at 0.8 ng/ml, 50% stimulation at 2–3 ng/ml, Figure 2).

The different source of CML CFU-GM (BM or PB) did not influence their response to either GM-CSF or IL3.

CFU-GM survival in liquid culture

Normal CFU-GM from 11 BM and two leukapheresis samples, as well as CML progenitors from 21 patients (either BM or PB samples) were tested for their capability to survive growth factor deprivation in liquid cultures.

The first 14 CML and seven control samples were tested after partial $CD34^+$ cell enrichment by negative selection. A steady decline of normal CFU-GM viability was observed (mean recovery: 55 ± 25% of day 0 concentration at the 4th day, 41 ± 18% at the 7th, 29 ± 15% at the 11th) (Table 1). Conversely, the average recovery of CML CFU-GM reached 124 ± 27%, 120 ± 46% and 117 ± 45% at day 4, 7, 11, respectively (Table 1). In particular, CFU-GM concentration remained stable or actually increased in 11/14 CML cases, whereas a slight decline (36–49%) was observed in 3/14 cases only. The difference in CFU-GM recovery between normal and CML samples was highly significant ($P = 0.02$ at days 4 and 11, $P = 0.004$ at day 7).

In experiments with highly purified progenitor cells from six control cases (median value of $CD34^+$ cells 77%), CFU-GM decay was even more evident, particularly in serum-free cultures. Indeed, the mean progenitor recovery at the 4th, 7th and 11th day was 28 ± 13%, 18 ± 10% and 16 ± 12%, respectively, in cultures with 10% FBS and 10 ± 12%, 4 ± 5% and 2 ± 4%, respectively, in serum-free medium (Table 1). By contrast, CML CFU-GM from seven cases survived well after extensive purification (median 94% $CD34^+$ cells), with average recovery, at the 7th and 11th day, of 92 ± 27% and 96 ± 37%, respectively (recovery above 75% in every case), in FBS containing cultures ($P = 0.008$ from controls) and 81 ± 17% and 67 ± 8%, respectively, in serum-free medium (three cases, $P = 0.05$ from controls) (Table 1).

Limiting dilution cultures

When highly purified $CD34^+$ cells were cultured in limiting dilution, a quite similar CFU-GM decay was detected in control (day 7 recovery: 36 ± 15% in 10% FBS, 7 ± 7% in serum-free) and in CML cases (day 7 recovery: 45 ± 28% in 10% FBS, 20 ± 13% in serum-free) (Table 1) ($P = 0.12$–0.8).

Table 1 Survival of normal and CML CFU-GM to growth factor deprivation in different culture conditions

Cells	*CD34⁺ cell enrichment*[a]	*Culture conditions*	*% CFU-GM recovery*[b]		
			Day 4	*Day 7*	*Day 11*
Normal	Low	FBS 10%	55 ± 25	41 ± 18	29 ± 15
Normal	High	FBS 10%	28 ± 13	18 ± 10	16 ± 12
Normal	High	Serum-free	10 ± 12	4 ± 5	2 ± 4
CML	Low	FBS 10%	124 ± 27	120 ± 46	117 ± 45
CML	High	FBS 10%	100 ± 34	92 ± 27	96 ± 37
CML	High	Serum-free	88 ± 16	81 ± 17	67 ± 8
Normal	High	FBS 10% Limit.dilution	49 ± 28	36 ± 15	ND
Normal	High	Serum-free Limit.dilution	12 ± 8	7 ± 7	ND
CML	High	FBS 10% Limit.dilution	59 ± 12	45 ± 28	ND
CML	High	Serum-free Limit.dilution	22 ± 19	20 ± 13	ND

Normal and CML cells, after different degree of CD34⁺ cell enrichment, were cultured for 7–11 days, either in mass culture or at limiting dilution, in IMDM + 10% FBS and in serum-free medium. At the start of the culture and after 4, 7 and 11 days CFU-GM concentration in mass culture was determined by plating in agar medium 50–100 μl of cell suspensions, as described in Materials and methods. For limiting-dilution assay, highly purified CD34⁺ cells were cultured at 1–10 cells/round bottom microwell in 50 μl of either IMDM + 10% FBS or serum-free medium. FBS and 5637 SN were added, in different plates, at day 0, 4, and 7 (see Materials and methods); 14 days later microwells were scored for clone growth and CFU-GM concentration was calculated by Poisson' s statistics on the basis of the proportion of growth-negative wells.[37]

[a]Low CD34⁺ cell enrichment refers to cases whose CD34⁺ cells were concentrated by removal of CD34⁻ cells (negative selection); high CD34⁺ cell enrichment refers to positive CD34⁺ separation by Miltenyi Minimacs.

[b]% CFU-GM survival, evaluated by comparing CFU-GM concentration in cell suspensions at day 0 and after 4–11 days of culture.

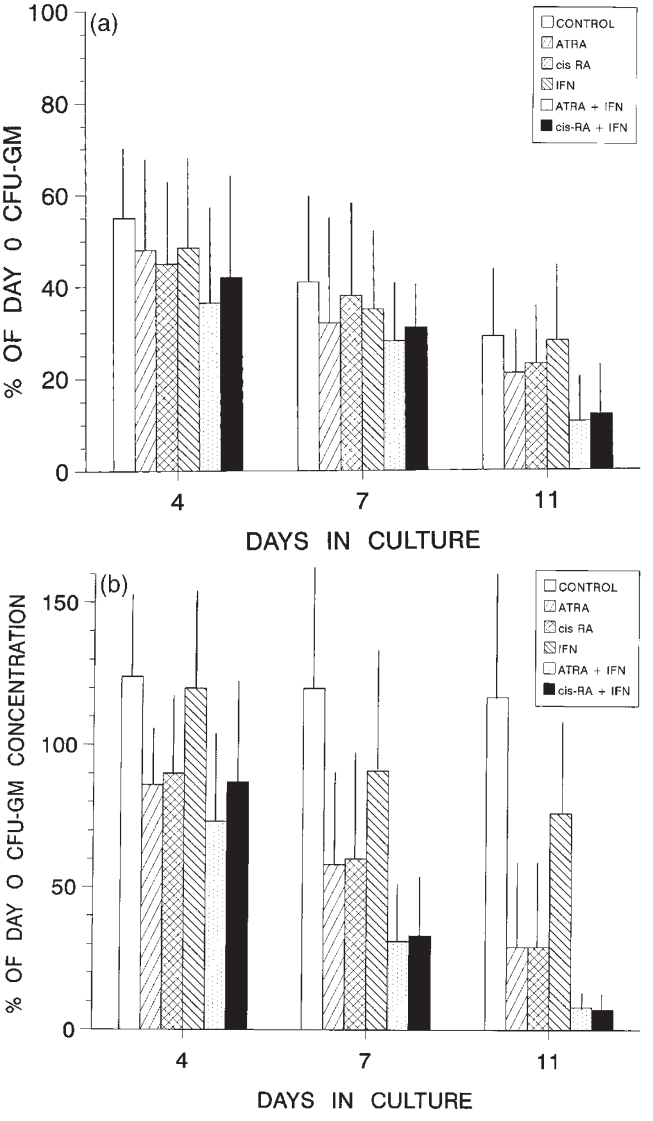

Figure 3 Effects of retinoids ± αIFN on survival of normal (a) and CML (b) CFU-GM in liquid culture. Hematopoietic progenitor-enriched cells were cultured in IMDM + 10% FBS, without exogenous growth factor, with/without retinoids and αIFN. At the start of the culture and after 4, 7 and 11 days, 50–100 μl of each cell suspension were harvested and plated in agar medium to determine CFU-GM concentration, as described in Materials and methods. CFU-GM survival was evaluated by comparing their concentration in cell suspensions at day 0 and after 4, 7, 11 days of liquid culture. CFU-GM concentration is expressed as mean percentage ± s.d. of day 0 value and illustrated in the Figure as vertical bars.

Retinoids/αIFN effect on CFU-GM survival

Retinoids and αIFN were tested on 17 CML cases (in 14 after low CD34⁺ cell enrichment, in three after high purification) and on six normal cases (after low CD34⁺ cell enrichment). Both αIFN, ATRA and *cis*RA, singularly used, did not affect the decline of normal CFU-GM in liquid culture. The combination of either retinoid with αIFN slightly accelerated progenitor cell loss, CFU-GM recovery being 31 ± 10% (with ATRA + αIFN) and 28 ± 13% (with *cis*RA + αIFN) at day 7 and 11 ± 10% (with either retinoid + αIFN) at day 11 (Figure 3a). However, the difference from controls (CFU-GM recovery 41 ± 18% at day 7, 29 ± 15% at day 11) did not reach statistical significance in the six experiments performed. In three preliminary experiments retinoids ± IFN were tested in cultures of unseparated low density normal BM cells, that allowed a better CFU-GM maintenance in control culture (76 ± 30% at day 7): in those conditions too, ATRA and cisRA evidenced only minimal suppressive activity on CFU-GM recovery (–10 ± 26% and –14 ± 30%, respectively, compared to control cultures, when used alone, –33 ± 10% and –31 ± 19% in combination with αIFN: those differences are not statistically significant).

Viability of CML CFU-GM (Figure 3b) was impaired in the presence of either ATRA or *cis*RA, with mean recoveries of 58 ± 32% and 60 ± 38%, respectively, at day 7 and 29 ± 33% and 29 ± 36%, respectively, at day 11. The differences from CFU-GM recovery in control cultures (–52% and –50%, respectively, at day 7, –75% at day 11) were significant ($P < 0.01$ at day 7, and <0.001 at day 11). Suppression of CFU-GM recovery was particularly evident when ATRA and *cis*RA were combined with αIFN, with mean recoveries of 31 ± 20% and 33 ± 23%, respectively, at day 7 (–74% and –72%, $P < 0.001$, compared to control cultures), 8 ± 8% and 7 ± 6%, respectively, at day 11 (–93% and –94% compared to control cultures, $P < 0.001$). In particular, in the presence of either retinoid + αIFN for 7 or more days, the decline of CML CFU-GM was comparable to that of normal ones in the

same culture conditions. Conversely, little, not significant inhibition was exerted by αIFN alone. The pattern of CML CFU-GM suppression by retinoids ± IFN was comparable after low or high CD34+ cell enrichment.

By considering single cases of CFU-GM recovery after 7–11 days, in drug-treated compared to control cultures, a greater than 50% inhibition could be detected in 12/17, 10/15, 1/16, 16/17 and 13/15 cases in the presence of ATRA, *cis*RA, αIFN, ATRA + αIFN and *cis*RA + αIFN, respectively. Table 2 illustrates day 7 CFU-GM recovery in the different culture conditions.

No correlation was found between sensitivity to retinoids ± αIFN and hyper-responsiveness to GM-CSF/IL3 or response to αIFN therapy.

Discussion

The first aim of our study was to verify whether some effects of bcr/abl fusion protein in IL3-dependent myeloid cell lines (ie CSF-independent survival and hypersensitivity to IL3-induced proliferation) might also apply to CML CFU-GM.

All patients studied had relevant leukocytosis and undetectable cytogenetically normal cells. However, to further minimise the chance of collecting residual normal progenitors, PB cells were used in most experiments. Indeed, CFU-GM frequency is quite higher in CML than in normal PB;[38] moreover, PB was demonstrated to be more apt than BM in maintaining long-term growth of CML progenitors.[39]

In order to minimise paracrine CSF production by accessory cells, in most experiments CFU-GM were enriched by removal of erythroid, lymphoid and mature myeloid cells. This method was initially preferred to the more efficient positive CD34+ cell selection to avoid any possible interference of CD34 MoAb binding on CFU-GM survival and growth. Then, the results obtained have been confirmed on positively selected, purified CD34+ cells.

Hypersensitivity of CML progenitor cells to GM-CSF and/or IL3 was not detected in previous studies involving nine and five cases, respectively;[30,14] conversely CML progenitors were recently found to display a higher than normal response to 'stem cell factor'.[40] In our study, a distinct hypersensitivity to IL3 and/or GM-CSF was evident in a minority of cases tested. The low proportion of hyper-responsive cases may explain the lack of such a feature in previous studies, involving relatively few CML cases. The biological significance of hypersensitivity to CSFs is not yet clear.

An increased capability of CML granulocytes[14] and CFU-GM[14,27] to survive *in vitro* to growth factor and FBS deprivation was already reported. However, conflicting results were provided by other studies.[28,29] The reasons of that discrepancy are unknown: it may be related to differences in culture medium composition or in the degree of accessory (CSF-producing) cell removal. In our study, CML CFU-GM survived well for 7–11 days in mass culture without CSF addition, even when purified CD34+ cells, and serum-free medium were employed.

CML BM cells have been reported to not sustain long-term cultures, allowing the emergence of residual normal progenitors.[41] However, those findings do not contrast to our results of a longer CML CFU-GM survival in culture, since CML cell depletion occurs after a few weeks on BM stroma, mainly involving stroma-adherent, early progenitors.[41,42] Moreover, our stroma-free cultures did not sustain normal myelopoiesis.

In contrast to results obtained in 'mass' culture, CML CFU-GM did not survive better than normal ones when purified CD34+ cells were cultured, by limiting dilution, at such low concentrations (1–10/50 μl) to greatly reduce the chance of autocrine/paracrine stimulation. This implies a cell concentration dependence of CFU-GM resistance to growth factor deprivation. Theoretically, the small proportion of CD34− cells still present in 'mass' culture after positive CD34+ cell selection (median 6%) might still contain some CSF-producing cells. However, this does not explain the results obtained,

Table 2 Response of CML cases to the inhibitory effect of retinoids ± αIFN on CFU-GM survival

Case No.	*CFU-GM/100 μl in the presence of:*					
	Medium	*ATRA*	*cisRA*	*IFN*	*ATRA + IFN*	*cisRA + IFN*
1	70 ± 1	**33 ± 1**	ND	70 ± 4	**25 ± 4**	ND
2	26 ± 8	**5 ± 1**	**3 ± 1**	ND	**0 ± 0**	**1 ± 1**
3	53 ± 5	58 ± 23	46 ± 15	37 ± 1	28 ± 11	31 ± 2
4	12 ± 1	**1 ± 1**	**3 ± 1**	8 ± 2	**1 ± 1**	**1 ± 1**
5	204 ± 11	182[a] ± 16	189[a] ± 9	206 ± 17	143[a] ± 21	138[a] ± 3
6	45 ± 1	**13 ± 10**	**9 ± 2**	37 ± 5	**8 ± 3**	**10 ± 1**
7	74 ± 11	**36 ± 13**	**37 ± 3**	58 ± 2	**15 ± 5**	**17 ± 9**
8	65 ± 14	36 ± 4	41 ± 8	51 ± 5	**15 ± 3**	**23 ± 1**
9	116 ± 8	**48 ± 3**	**35 ± 13**	**52 ± 9**	**33 ± 1**	**17 ± 5**
10	17 ± 2	**7 ± 1**	13[a] ± 2	15 ± 1	**4 ± 1**	**7 ± 1**
11	50 ± 1	47 ± 2	45 ± 2	41 ± 8	**23 ± 15**	30 ± 2
12	358 ± 3	270[a] ± 20	307 ± 15	296 ± 50	**121 ± 13**	**105 ± 6**
13	357 ± 5	**177 ± 42**	263[a] ± 125	255 ± 106	**97 ± 1**	**53 ± 56**
14	71 ± 24	64 ± 8	60 ± 8	59 ± 4	**34 ± 0**	41[a] ± 8
15	285 ± 85	**52 ± 17**	**17 ± 6**	227 ± 82	**5 ± 2**	**40 ± 3**
16	303 ± 46	**73 ± 8**	**61 ± 5**	236 ± 54	**21 ± 4**	**61 ± 9**
17	111 ± 4	67 ± 7	ND	76 ± 30	62[a] ± 15	ND

CML cells from 17 patients were cultured in IMDM + 10% FBS, in the presence/absence of retinoids and/or αIFN, as described in Materials and methods. CFU-GM concentration after 7 days of culture (evaluated as described in Materials and methods) is here illustrated. Values are mean ± s.d. of duplicated liquid cultures. Bold numbers refer to cultures with CFU-GM concentration <50% of control culture value. [a]Cultures where a greater than 50% inhibition of CFU-GM recovery was detected at day 11 only. ND, not determined.

since even higher proportions of $CD34^-$ cells did not allow normal CFU-GM maintenance. Moreover, equal CFU-GM survival was detected in CML cases with 80–85% and >98% $CD34^+$ cells, respectively. Conversely, CML $CD34^+$ cells have recently been described to auto-produce IL3 and G-CSF.[26] Therefore, enough CSFs to allow CFU-GM survival could be released in the presence of a relatively high but not at very low cell concentration. As an alternative hypothesis, autocrine CSF production could be restricted to a fraction of CML progenitors capable of expanding autonomously[26] and replacing other CFU-GM unable to survive, thus maintaining their final concentration. This could not happen when only one or very few CFU-GM are plated in limiting dilution cultures. The same might occur should the majority of CD34 cells be represented by residual normal progenitors, with few leukemic ones capable of survival and proliferation. However, this seems very unlikely, as patients presented with florid disease and no normal mitosis detected; therefore, residual normal progenitors, whether present, should have be confined among very early (HLA-DR negative) $CD34^+$ cells,[43,44] representing a minimal proportion (<1%) of total $CD34^+$.

As first conclusions of our study, hypersensitivity to GM-CSF or IL3 proliferative stimulus looks an uncommon feature of CML CFU-GM, that, conversely, display a cell concentration-dependent resistance to growth factor deprivation, possibly related to autocrine CSF production. This feature seems strictly related to the activity of BCR/ABL fusion protein, since a recent report[45] described a suppression of CML progenitor survival by STI571, a powerful inhibitor of bcr/abl tyrosine kinase.[46]

A further purpose of our experiments was to test the effects of retinoids and αIFN on CML CFU-GM. ATRA was already reported to inhibit *in vitro* colony formation by CML CFU-GM[47–49] and its suppressive activity to be enhanced by αIFN.[48,49] We wished here to test whether ATRA and 13-*cis* RA ± αIFN, at therapeutic concentrations, could suppress the survival advantage of CML CFU-GM in growth factor-deprived cultures. Indeed, most CML cases displayed sensitivity to the suppressive effect of both ATRA and 13-*cis* RA, without significant difference in activity between the two stereo-isomers. Both the percentage of responsive cases and the degree of inhibition increased in the presence of either retinoid + αIFN, whose combination completely overcame CML survival advantage. The suppression of CFU-GM recovery by retinoids ± αIFN was only evident after more than 4 days of exposure. This is in contrast to results of another group[50] who reported the ability of 24 hour exposure to ATRA + αIFN to suppress CML and normal CFU-GM. One possible explanation for that discrepancy may rely on the higher αIFN concentration used (1000 U/ml) in that study, which proved to be quite toxic for normal CFU-GM too.

In a mouse model, ATRA was recently demonstrated to be capable of expanding normal primitive hematopoietic progenitors and enhancing the granulocytic differentiation of late CFU-GM.[51] In our present experiments and previous reports[52,53] retinoids ± αIFN evidenced only minimal suppression of normal CFU-GM maintenance. These data and retinoid activity on CML CFU-GM support a possible role of retinoids in CML therapy. Indeed, while retinoids alone have been proven of little benefit,[54,55] the combination with αIFN looks promising.[56] Our experiments also showed that 13-*cis* RA had the same activity on CML progenitors as ATRA; in agreement with other reports.[49,56] This could have clinical relevance since 13-*cis* RA has a better pharmacokinetic profile than ATRA, with more stable stable plasma levels during continuous administration.[55,57] Presently available *in vitro* results can probably justify clinical trials of retinoid, combined with αIFN, in CML treatment.

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