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INTERLEUKIN-1 AND INTERLEUKIN-2 CONTROL GRANULOCYTE- AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR GENE EXPRESSION AND CELL PROLIFERATION IN CULTURED ACUTE MYELOBLASTIC LEUKEMIA

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***In vitro* proliferation of leukemic cells purified from 10 cases of acute myeloblastic leukemia (AML) was analyzed in basal conditions or in the presence of exogenous recombinant (r) Interleukin (IL) 1. In parallel, blasts from 5 of these patients were studied for granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte-CSF (G-CSF) mRNA. IL-1 augmented the spontaneous AML cell proliferation in all cases and induced *de novo* expression or increased amounts of GM-CSF and/or G-CSF transcripts in 4 of the 5 cases evaluated. IL-1-induced AML cell proliferation was modulated by neutralizing anti-GM-CSF or anti-G-CSF antibodies in those cases in which CSF mRNAs were induced or increased by exogenous cytokine. In the same cases, biosynthetic labelling and immunoprecipitation studies using monospecific anti-GM-CSF antibodies showed that IL-1 also increased the levels of GM-CSF protein synthesis. Addition of neutralizing anti-IL-1 antibodies to AML cell cultures completely abolished ongoing GM-CSF synthesis, suggesting that endogenous IL-1 is needed to maintain autocrine production of CSFs. The effects of rIL-2 were investigated in a larger series of 21 patients. The cytokine reduced spontaneous AML cell proliferation in 8 cases. It caused complete disappearance of GM-CSF mRNA in 1 case, and marked reduction of G-CSF mRNA in 2 cases. Increased AML cell proliferation was observed in 2 of 21 cases. These findings suggest that expression of CSF genes and cell proliferation in AML are under the control of different cytokines acting in autocrine or paracrine fashion.**

Normal hematopoiesis is regulated through discrete steps of differentiation, which require the action of growth factors, and particularly a group of glycoproteins known as colony-stimulating factors (CSFs) (Clark and Kamen, 1987). Growth of normal and leukemic blast progenitors is also influenced by the activity of a growing number of other molecules. In particular, cytokines, originally thought to mainly regulate immune functions or inflammation, such as γ-Interferon (γ-IFN), tumor necrosis factor-α (TNF-α), Interleukin (IL)-1, IL-4 and IL-6, have been shown to play a critical role in hematopoiesis (Metcalf, 1989; Trinchieri *et al.*, 1987). Indeed, these proteins are linked in a complex network, whereby they influence each other's function, in terms of gene expression, protein synthesis and secretion, and interaction with surface receptors. Further complexity is added by the observation that CSFs and IL-1 genes are constitutively expressed in acute myeloblastic leukemia (AML) cells, with production and secretion of the respective mature proteins (Young and Griffin, 1986; Young *et al.*, 1987; Rambaldi *et al.*, 1988; Griffin *et al.*, 1987; Furukawa *et al.*, 1987; Cozzolino *et al.*, 1988, 1989). It is possible that the factors are linked by regulatory connections within the leukemic cell. Surface expression of IL-2 receptor light chain has been reported in AML (Hermann *et al.*, 1985; Rambaldi *et al.*, 1987). Although functional consequences of leukemic cell interactions with the cytokine have not been defined, studies on normal monocytes have demonstrated an influence on some cellular functions (Malkovsky *et al.*, 1987). Dissection of such interactions appears important for a thorough understanding of normal as well as of leukemic hematopoiesis.

We therefore decided to investigate the effects of 2 cytokines, IL-1 and IL-2, on AML cell proliferation and on gene expression and protein synthesis of GM-CSF and G-CSF.

MATERIAL AND METHODS

Leukemic cells

Leukemic cells were obtained, after informed consent, at diagnosis from peripheral blood of 21 randomly selected patients and the mononuclear fraction was isolated by sedimentation on Ficoll-Hypaque density gradients. Samples were cryopreserved in 10% DMSO in the vapor phase of liquid nitrogen until use. The diagnosis of AML was established by morphology, cytochemistry and surface marker analysis using a panel of anti-myeloid monoclonal antibodies (MAbs) (Griffin *et al.*, 1981). The patients were classified according to the FAB scheme (Bennett *et al.*, 1976) as follows: 4 patients with M1 AML, 6 with M2, 6 with M4, and 5 with M5. In all experiments, leukemic cells were depleted of T cells by rosetting with neuraminidase-treated sheep red blood cells and depleted of monocytes by plastic adherence at 37° C. The percentage of cells expressing the p55 subunit of IL-2 receptor was assessed by flow cytometry of cells stained with anti-CD25 MAbs (anti-Tac) (Cozzolino *et al.*, 1987).

Recombinant cytokines and antibodies

Purified human recombinant (hr) IL-1 α and β (specific activity of both: 1.3×10^7 half maximal units/mg in the thymocyte co-stimulation assay) and hrIL-2 (specific activity: 1.3×10^7 units/mg in the CTLL-2 proliferation assay) were obtained through the courtesy of Dr. A. Shaw (Glaxo, Geneva, Switzerland). IgG fractions of neutralizing anti-GM-CSF, anti-G-CSF, anti-IL-1 α and anti-IL-1 β sheep antisera, generous gifts from Dr. S. Poole (National Institute for Biological Standards and Controls, Potters Bar, UK), and of pre-immune sheep or rabbit serum were obtained by Affi-Gel Protein A (Bio-Rad, Milan, Italy) affinity chromatography and used at 5 μg/ml final dilution for proliferation or immunoprecipitation studies. The latter concentration of anti-CSFs antibodies neutralized the growth-promoting activity of 250 U/ml of rGM-CSF or G-CSF (Genzyme, Boston, MA) on selected AML cells; 5 μg of anti-IL-1 α or anti-IL-1 β IgG neutralized >15 ng of the respective cytokine, with no cross-reactivity, in the

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thymocyte co-stimulation assay. Neutralizing rabbit anti-human IL-2 IgG (specific activity: 3000 neutralizing units/mg) were purchased from Collaborative Research (Bedford, MA) and used at 50 μg/ml final dilution.

Leukemic cell proliferation assay

For the cell proliferation studies, different concentrations of leukemic cells, from 5×10^4/ml to 2.5×10^5/ml, were incubated in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS, GIBCO) for 48 hr, in the presence or absence of the different cytokines and of neutralizing antibodies or control IgG. Cultures were pulsed with 0.5 μCi of ^{3}H-thymidine (specific activity 25 Ci/mM, Amersham, Little Chalfont, UK) 12 hr before harvesting and radioactivity was determined in a liquid scintillation counter (Beckman Analytical, Milan, Italy).

Northern blots

Cells (5×10^7), cultured for 12 hr in the presence or absence of IL-1 or IL-2, 5 ng/ml each, were lysed in 4 M guanidinium isothiocyanate and RNA was extracted, electrophoresed in agarose gel, and blotted (Rambaldi *et al.*, 1988). Membranes were hybridized to GM-CSF and G-CSF cDNA probes (kindly provided by Drs. S. Clark and G. Wong, Genetics Institute, Cambridge, MA) (Clark and Kamen, 1987), labelled with ^{32}P-dCTP by the method of Feinberg and Vogelstein (1983). The UCD-MLA 144 gibbon cell line was obtained from ATCC (Rockville, MD) and grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum; after stimulation with 20 ng/ml phorbol myristate acetate (PMA, Sigma, St. Louis, MO), RNA was isolated from this cell line and used as a source of GM-CSF RNA. To ascertain that comparable amounts of RNA were transferred, each membrane was re-hybridized to a mouse actin cDNA probe.

Immunoprecipitation studies

Endogenous labelling and immunoprecipitation studies were performed as described by Cozzolino *et al.* (1989). Cells were incubated at 10^7/ml for 12 hr in methionine- and cysteine-free RPMI 1640 medium (GIBCO) supplemented with 5% dialyzed FCS, 5×10^{-5} M 2-mercaptoethanol (2-ME), ^{35}S-methionine and ^{35}S-cysteine (specific activity: 800 Ci/mmol and 600 Ci/mM, respectively, NEN-Du Pont, Florence, Italy), 100 μCi/ml each, in the presence or absence of rIL-1 α or β, 5 ng/ml. In selected experiments, cells were cultured with IgG fraction of neutralizing anti-IL-1 α and anti-IL-1 β antisera (5 μg/ml each), or of control sheep serum. Cells were then centrifuged, washed and lysed in PBS with 0.5% Nonidet P-40. Samples of supernatants and of cell lysates were immunoprecipitated with 5 μg of IgG fraction of anti-GM-CSF or of pre-immune sheep IgG as control, followed by 50 μl of Protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Immunoprecipitates were extensively washed, eluted by boiling in Laemmli stacking buffer containing 2% (v/v) SDS and 5% 2-ME, and subjected to SDS-polyacrylamide (12%) gel electrophoresis. Gels were then dried and autoradiographed with intensifying screens.

RESULTS

Effects of IL-1 and IL-2 on AML proliferation

We have previously shown that rIL-1 α or β induce *in vitro* growth of AML cells (Cozzolino *et al.*, 1989). To further investigate this point, the effect of IL-1 was studied in a proliferation assay (^{3}H-thymidine incorporation), using different concentrations of blast cells plated with the recombinant factor for 48 hr, with or without neutralizing anti-CSF antibodies. Table I summarizes the results obtained with cells from 10 AML patients, where IL-1 induced an increased proliferation rate at the appropriate cell density in culture (Cozzolino *et al.*, 1989). The addition of neutralizing antibodies directed against GM-CSF was followed by a significant, though not complete, reduction of IL-1-induced AML proliferation in 6 cases. When tested, neutralizing anti-G-CSF antibodies showed a pattern of activity parallel to that of anti-GM-CSF antibodies.

TABLE I – EFFECT OF IL-1 ON AML CELL PROLIFERATION

Case number	^{3}H-TdR incorporation (cpm) in the presence of[1]			
	Medium	IL-1[2]	IL-1 + anti-GM-CSF[2,3]	IL-1 + anti-G-CSF[2,3]
1	18,235	45,645	20,438	27,848
2	4,370	21,438	13,456	15,437
3	2,850	8,545	9,043	ND
4	10,368	51,543	18,775	21,348
5	2,235	30,456	4,004	32,043
6	764	7,843	7,935	ND
7	2,491	5,432	5,177	4,988
8	4,095	18,473	9,543	13,334
9	323	2,445	657	ND
10	723	6,148	6,044	5,980

[1]Leukemic cells were cultured at 2.5×10^5/ml (cases 1, 3, 4, 5), at 1.2×10^5/ml (cases 2, 6, 8, 10), or at 5×10^4/ml (cases 5, 7) for 48 hr in the presence or absence of the indicated reagents. ^{3}H-TdR was added during the last 12 hr of culture. Results are expressed as mean cpm of triplicate cultures. SD was consistently <10%. –[2]hrIL-1α or β were used at the final concentration of 5 ng/ml.–[3]Neutralizing sheep anti-GM-CSF and anti-G-CSF antibodies were used at the final concentration of 5 μg/ml. Pre-immune sheep IgG, used as control at the concentration of 5 μg/ml, was ineffective in the assay.

Based on the observation that myeloid leukemic cells, as well as activated normal monocytes, can express the p55 subunit of the IL-2 receptor on their surface (Herrmann *et al.*, 1985; Rambaldi *et al.*, 1987), we tested, in a larger series of cases, the possibility that IL-2 might also work on AML as a growth-regulating signal. When IL-2 was added to the culture medium, we observed in 8 of 21 cases variable degrees of inhibition of AML proliferation, ranging from 52 to a maximum of 83% (Table II). In 2 cases, however, definite induction of growth was recorded. The addition of saturating amounts of neutralizing antibodies directed against human IL-2 was able to prevent the activity of AML cell proliferation. Phenotypic analysis, performed on 13 of these cases, showed a correspondence between the response to IL-2 and the expression of the p55 subunit of the IL-2 receptor complex (Table II).

Effects of IL-1 and IL-2 on GM-CSF and G-CSF gene expression

Because CSFs have been reported to act as autocrine stimulators of AML blast proliferation (Young and Griffin, 1986; Young *et al.*, 1987; Rambaldi *et al.*, 1988; Griffin *et al.*, 1987), we tested the possibility that the effects of IL-1 and IL-2 on leukemic growth could be exerted, at least in part, through interference with expression of CSF genes, as suggested by the above experiments with IL-1 and anti-GM-CSF or anti-G-CSF antibodies. Upon culture in the presence of medium alone or with rIL-1β, leukemic-cell-derived mRNA was examined for GM-CSF and G-CSF gene expression (Fig. 1*a*, *b*). In one case (5) leukemic cells cultured in medium alone did not express any of the growth factor genes studied, but the addition of rIL-1 β was followed by the accumulation of GM-CSF mRNA within the blasts. In 3 cases (1, 2 and 4) expression of GM-CSF and G-CSF genes was detectable upon culture in medium alone. In all of these the exposure of leukemic blasts to IL-1 was followed by increased levels of GM-CSF transcription, whereas definite augmentation of G-CSF mRNA was induced only in blasts from case 1. In one more case (3), no GM-CSF or G-CSF transcripts were detected under control conditions, as well as after addition of IL-1. Remarkably, in single cases a close correspondence was observed between levels of CSF

TABLE II – EFFECT OF IL-2 ON AML CELL PROLIFERATION

Case number[4]	^{3}H-TdR incorporation (cpm) in the presence of[1]		
	Medium	rIL-2[2]	rIL-2[2] + anti-IL-2[3]
1	18,235	5,659	18,043
2	6,556	3,147	5,740
3	2,850	3,120	ND
4	10,368	9,675	11,243
5	2,235	703	1,772
6	1,252	1,374	1,278
7	8,538	8,525	ND
8	12,659	6,102	11,964
9	2,675	2,450	ND
10	1,348	1,426	ND
11	1,852	17,353	2,657
12	2,491	2,626	ND
13	564	871	ND
14	517	630	ND
15	22,238	14,558	23,467
16	1,160	202	1,122
17	12,595	33,189	14,236
18	720	690	ND
19	1,314	457	1,103
20	47,192	35,190	46,883
21	9,747	10,449	ND

[1]Leukemic cells were cultured at 2.5×10^5/ml for 48 hr. ^{3}H-TdR was added during the last 12 hr of culture. Results are expressed as mean cpm of triplicate cultures. SD was consistently <10%.–[2]rIL-2 was used at 5 ng/ml.–[3]Rabbit neutralizing anti-IL-2 antibodies were used at the final concentration of 50 μg/ml. Preimmune rabbit IgG, used as control at the same concentration, was ineffective in the assay.–[4]Cases 1, 2, 5, 8, 11, 15 and 20 had >80% circulating CD25$^+$ cells; cases 3, 4, 6, 7, 9 and 10 had <10% CD25$^+$ cells. ND: not determined.

gene expression in response to IL-1 and modulation of IL-1-induced proliferation by anti-CSF antibodies (Fig. 1, Table I).

By contrast, exposure of leukemic blasts from case 1 to IL-2 was followed by complete suppression of high levels of GM-CSF mRNA constitutively present under control conditions (Fig. 1*a*). While no significant modulation of GM-CSF transcription was found in cases 2 and 4, the inhibitory effect of IL-2 on G-CSF gene expression was observed in cases 1 and 2 (Fig. 1*b*). As shown in Table I, in these latter cases rIL-2 caused a reduction in spontaneous leukemic cell proliferation. However, this occurred also in case 5, where no constitutive expression of CSF genes was detected.

Effects of rIL-1 and of anti-IL-1 antibodies on GM-CSF production

In order to verify whether leukemic cells were indeed synthesizing GM-CSF protein, control and IL-1-treated blasts, metabolically labelled with ^{35}S-methionine and ^{35}S-cysteine, were tested for expression of the protein using polyclonal anti-GM-CSF antibodies. As shown in Figure 2, the 25-kDa polypeptide was immunoprecipitated from leukemic cell lysates of case 2, with significantly higher levels of protein in IL-1-treated blasts. No GM-CSF was detected in culture supernatants. The same pattern was observed in cases 1 and 4 (not shown).

We have previously shown that IL-1 is invariably produced by AML cells, and that neutralizing anti-IL-1 antibodies affect leukemic cell proliferation (Cozzolino *et al.*, 1989). To ascertain whether endogenous IL-1 sustains autocrine GM-CSF production, at least in those cases with constitutive synthesis, in another series of experiments, AML cells from case 1 were metabolically labelled upon culture with neutralizing anti-IL-1 α and anti-IL-1 β antibodies, and cell lysates were studied as above. Figure 3 shows that incubation with anti-IL-1 antibodies, in amounts sufficient to abrogate leukemic cell proliferation, completely abolished GM-CSF synthesis in culture, a finding which further supports the hypothesis that IL-1 and CSFs are functionally linked within the malignant cell.

DISCUSSION

Proliferation of human leukemic myeloblasts requires the presence of CSFs (Clark and Kamen, 1987; Metcalf, 1989; Trinchieri *et al.*, 1987). Evidence has been provided on disregulated CSF gene expression which, in some cases of AML, could sustain autocrine stimulation (Vellenga and Griffin, 1987). However, other cases of AML show no apparent CSF gene activation, and a more sophisticated network of humoral interactions between endothelial cells, fibroblasts and leukemic cells could be responsible for growth factor supply. Part of this interaction could be mediated by biologically active IL-1 which is secreted by the majority of AMLs and is a potent stimulator of CSF secretion by fibroblasts and endothelial cells (Griffin *et al.*, 1987; Seelentag *et al.*, 1989; Sieff *et al.*, 1987). On the other hand, a direct IL-1-mediated autocrine stimulation pathway in AML has also been proposed (Cozzolino *et al.*, 1989).

In the present work we show data suggesting that IL-1 can stimulate AML growth *in vitro* through its ability to regulate gene expression and protein synthesis of GM-CSF and G-CSF. As already seen (Cozzolino *et al.*, 1989), IL-1 was able to induce leukemic cell proliferation, as determined by ^{3}H-thymidine incorporation; such growth factor activity was inhibited partially, though not completely, by the addition to the culture media of anti-GM-CSF or anti-G-CSF polyclonal antibodies, thus supporting the hypothesis that at least part of the effect is mediated by these CSFs. Using Northern blot analysis we have shown that, when exposed to IL-1, GM-CSF or G-CSF transcription could be induced or up-regulated in the majority of the AMLs cases studied. Under the same culture conditions, this finding was confirmed by the immunoprecipitation of increased levels of GM-CSF protein. These results confirm and extend those of Delwel *et al.* (1989). Abrogation of IL-1-induced proliferation by anti-GM-CSF antibodies was not complete. This finding suggests the induction of growth factors other than those tested. However, this observation, together with the detection in our cases of GM-CSF in cell lysates but not in culture supernatants, could also be explained considering that the growth factor delivers at least part of its proliferative signal within intracellular compartments, as demonstrated in leukemic cell lines transfected with modified IL-3-coding genes (Dunbar *et al.*, 1989). The notion that IL-1 can subserve autocrine functions in AML raises the question of whether the endogenous cytokine is directly exerting its growth-promoting activity or whether it regulates the levels of other autocrine factors, possibly sustaining neoplastic growth. The observation that rIL-1 increases endogenous CSF levels, while anti-IL-1 antibodies abrogate their synthesis, favors the latter hypothesis, that of a hierarchical connection linking the factors. However, it is conceivable that expression of other genes, such as IL-3 or IL-6, is up-regulated by IL-1, as suggested by cytokine-induced proliferation in those cases in which no GM-CSF or G-CSF transcripts were detected. The question bears relevance for possible therapeutic strategies.

AML cells may express the p55 sub-unit of the IL-2 receptor (Herrmann *et al.*, 1985; Rambaldi *et al.*, 1987). Our results show that incubation with rIL-2 induces a decrease in autonomous cell proliferation in 8 of 21 AML cases. In 2 cases, characterized by high levels of Tac$^+$ blasts, autonomous growth *in vitro*, and constitutive expression of the GM-CSF and/or G-CSF genes, incubation with rIL-2 caused a reduction in the proliferation rate and a decrease or even abrogation of CSF gene expression. In one case with no expression of either G-CSF or GM-CSF, IL-2 had no effect on blast proliferation;

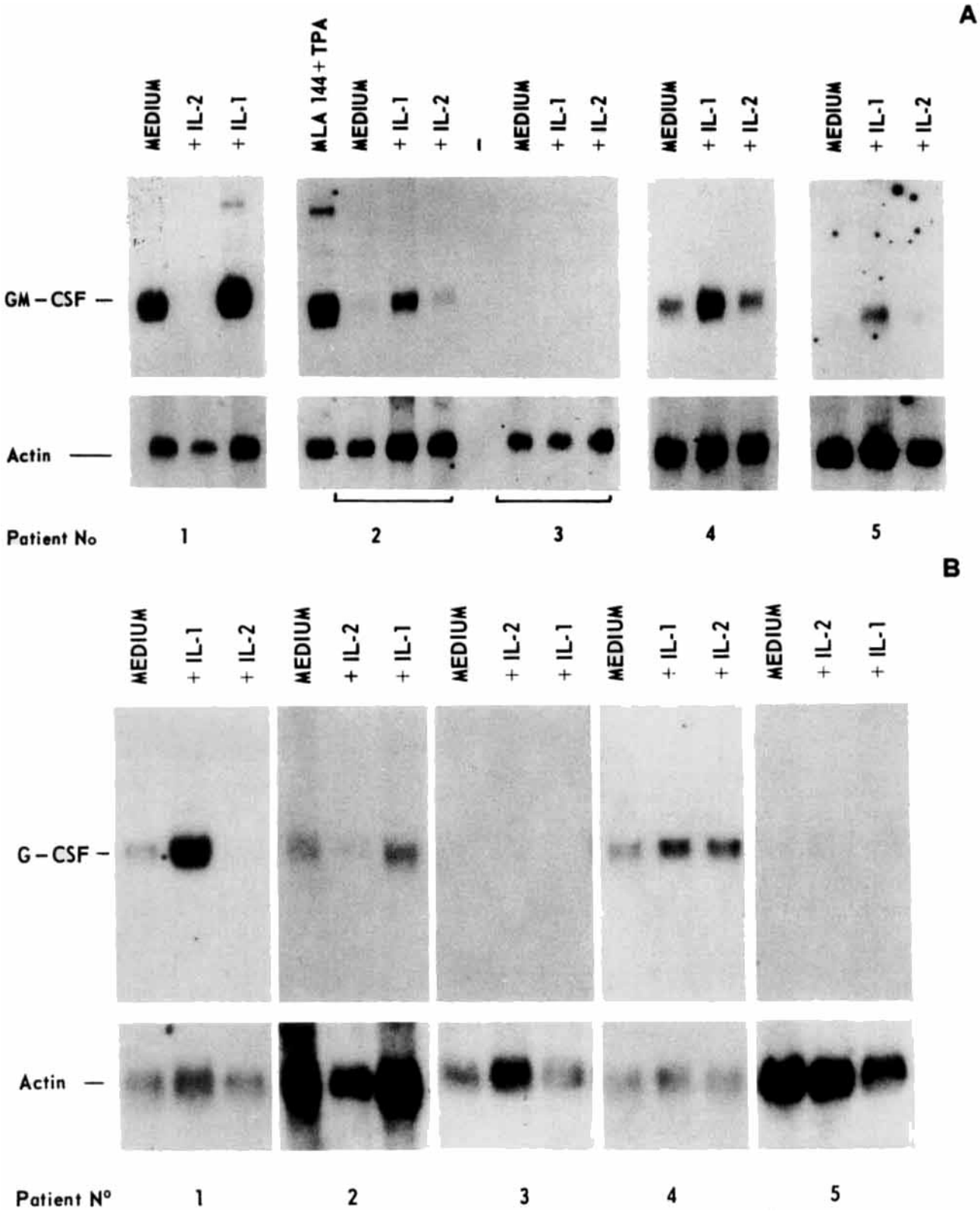

FIGURE 1 – Effects of IL-1 and IL-2 on GM-CSF *(a)* or G-CSF *(b)* gene expression. Northern blot of mRNA extracted from 5×10^7 cells of cases 1–5, cultured for 12 hr with or without 5 ng/ml of either cytokine. The amount of mRNA loaded into each lane was checked by rehybridizing the membrane to a mouse actin cDNA probe.

in another case with similar features the cytokine profoundly reduced leukemic cell growth. This might be explained by interference with the expression of other growth factors or of surface receptors. Our data are consistent with the reported maturation- rather than growth-promoting effects of IL-2 on normal monocytes. However, in 2 cases, induction of prolif-

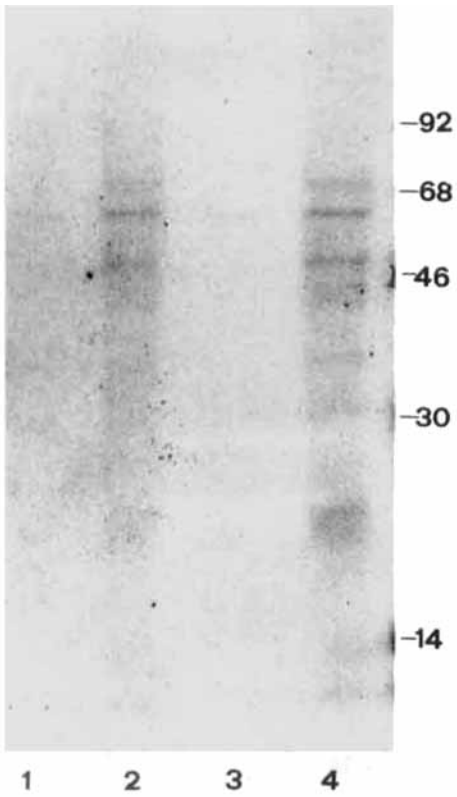

FIGURE 2 – Effect of IL-1 on GM-CSF protein synthesis. Cells from case 2 were metabolically labelled in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 5 ng/ml of rIL-1 β, and supernatants (lanes 1 and 3) and cell lysates (lanes 2 and 4) were immunoprecipitated with anti-GM-CSF polyclonal antibodies, run in SDS-polyacrylamide gel electrophoresis and autoradiographed.

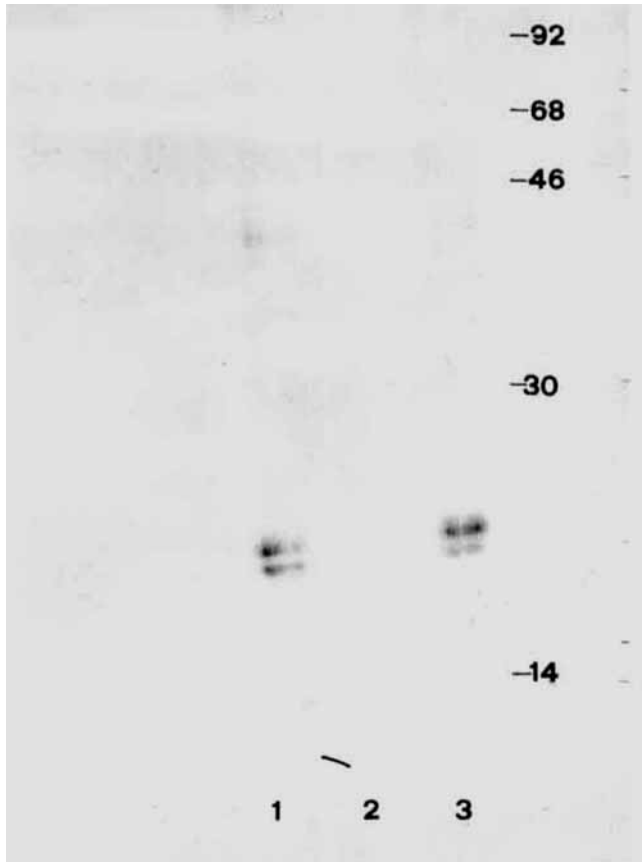

FIGURE 3 – Effect of anti-IL-1 antibodies on GM-CSF protein synthesis. Cells from case 1 were metabolically labelled upon culture in medium alone (lane 1), in the presence of 5 μg/ml of neutralizing sheep anti-IL-1 polyclonal IgG (lane 2), or of 5 μg/ml of sheep preimmune IgG (lane 3). Cell lysates were immunoprecipitated with anti-GM-CSF antibodies, run in SDS-polyacrylamide gel electrophoresis and autoradiographed.

eration has been noted, as also observed, although with a higher frequency, by Carron and Cawley (1989). The functional heterogeneity of IL-2 activity probably reflects its impingement on undefined, subtle differences in the molecular pathways governing AML cell proliferation and differentiation. For example, it is possible that in certain AML cases IL-2 induces IL-1 secretion, as reported for normal monocytes (Hermann *et al.*, 1989), thus boosting cell growth. Overall, these findings suggest that IL-2 may also be involved in the mechanisms controlling proliferation of normal myeloid progenitor cells. Finally, the observation that the cytokine *per se* affects malignant cell proliferation, albeit in a minority of cases, could also have a clinical relevance, in the light of ongoing therapeutic trials using lymphokine-activated killer (LAK) cells plus rIL-2.

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