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Research Article

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In Vivo Differentiation of Blast-phase Chronic Granulocytic Leukemia

Expression of *c-myc* and *c-abl* Protooncogenes

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Abstract

A patient with chronic granulocytic leukemia in acute blastic transformation was treated with mithramycin, an RNA synthesis inhibitor, after in vitro exposure of her leukemic cells to mithramycin showed differentiation to normal appearing granulocytes. Mithramycin therapy in vivo resulted in a prompt and dramatic hematologic response. Before therapy, the patient's leukemic cells had high levels of transcription of the cellular *myc* and *abl* protooncogenes. After initiation of therapy, protooncogene mRNA decreased rapidly. These observations indicate that mithramycin can induce differentiation both in vitro and in vivo and suggest that such changes may be associated with altered oncogene expression.

Introduction

Chronic granulocytic leukemia (CGL)[1] is a myeloproliferative disorder associated in >90% of cases with the Philadelphia chromosome (Ph^1) [t(9;22)(q34;q11)] (1, 2). After a variable period of time, the majority of patients enter a terminal phase that may resemble an acute blastic leukemia (blast crisis). About two-thirds of blast-phase CGLs have recognizable myeloblast or promyelocyte morphology but differ from *de novo* acute myelogenous leukemia in that they are rarely sensitive to cytotoxic chemotherapy. A differentiation model has been developed using leukemic cell lines that can be induced to differentiate in vitro into cells with the characteristics of normal hematopoietic end cells (3). Upon differentiation, the leukemic cells cease to proliferate and lose their ability to cause leukemia when transplanted into animals. A murine model also exists wherein the survival time of mice inoculated with syngeneic differentiation-sensitive leukemia cells is significantly improved if they are subsequently treated with a differentiation-inducing agent (4). A question of profound therapeutic importance is whether the leukemic myeloblasts in CGL can be induced to differentiate into functional granulocytes.

Several lines of evidence indicate that abnormal expression of oncogenes (5) may be important in the pathogenesis of CGL as well as other neoplasms (6, 7). A protooncogene, *c-abl*, the cellular homologue of the transforming sequence of the Abelson murine leukemia virus (8), is ordinarily located on chromosome 9q34 (9) and becomes translocated to chromosome 22 with the Ph^1 translocation (10). Another protooncogene of interest in CGL is *c-myc*, the cellular homologue of the transforming sequence of the avian myelocytomatosis virus MC29 (11). *C-myc* is located on chromosome 8 (12) and is involved in translocations involving chromosomes 14 (13) (heavy chain locus), 2, or 22 (light chain loci) in Burkitt's lymphoma cells (14, 15). The *c-myc* gene is highly amplified in the human promyelocytic cell line HL60 (16), as well as in other cell lines (17, 18), resulting in raised levels of *c-myc* mRNA. *C-myc* has also been found to be transcriptionally active in normal hematopoietic tissue as well as in CGL cells (19, 20). Recent evidence indicates that the product *c-myc* encodes for is a nuclear protein capable of binding to DNA (21), and that levels of both *c-myc*–directed mRNA and *c-myc* protein are quickly increased after activation of cells with mitogens (22). While the function of the *c-myc* gene product is not yet clear, it has been postulated that it is involved in the control of cellular proliferation (21, 22).

We report here a patient with CGL who had an acute blast transformation associated with enhanced expression of *c-abl* and *c-myc* genes, whom we treated with mithramycin, an inhibitor of RNA synthesis (23). There was a prompt reduction in *c-abl* and *c-myc* mRNA transcripts, followed soon after by evidence of in vivo differentiation and a dramatic beneficial clinical response. These findings indicate that it is possible to inhibit oncogene expression in vivo, and that such action may be associated with clinical benefit.

Methods

Isolation of leukocytes and in vitro differentiation. Mononuclear cells were isolated from peripheral blood by Ficoll-Hypaque centrifugation (24). Washed mononuclear cells were split into two flasks containing RPMI-1640 plus 10% fetal calf serum (medium). Mithramycin (Mithracin; Miles Laboratories, Inc., West Haven, CT) was added to one flask at a concentration of 4.6×10^{-7} M for 4 h. The mithramycin was removed and the cells resuspended in mithramycin-free medium. At various time points, cells were harvested from each flask, stained with Wright-Giemsa stain, and differential white blood counts (WBC) performed.

RNA synthesis determination. Whole blood was diluted with an equal volume of medium. 25 μCi of [^{3}H]uridine was then added for

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1. *Abbreviations used in this paper:* CGL, chronic granulocytic leukemia; WBC, white blood count(s).

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30 min at 37°C. After precipitation with trichloroacetic acid, incorporation of the tritiated uridine into radiolabeled RNA was quantitated by filtration on GF/C glass filters (Whatman Inc., Clifton, NJ).

Quantitative RNA dot blots. RNA was isolated as previously described (25). Briefly, mononuclear cells were isolated from peripheral blood as described above. The washed cell suspension was extracted with phenol in the presence of vanadyl ribonucleoside and heparin (26). All operations were carried out at 4°C. RNA was further purified by cesium chloride centrifugation. Total cellular RNA was attached to nitrocellulose and hybridized as described by Thomas (27).

Cloned cDNA probes. The *c-abl* probe was supplied by Dr. David Baltimore, Massachusetts Institute of Technology, Cambridge, MA (28). The *c-myc* probe was obtained from Dr. Robert Gallo, National Cancer Institute, Bethesda, MD (29). The chicken lysozyme probe (*plys 6*) was obtained from Dr. Robert Shimke, Stanford University, Stanford, CA (30). The probes were labeled with ^{32}P by nick-translation to 2×10^8 cpm/μg sp act.

Results

Clinical summary. A previously healthy 18-yr-old woman was diagnosed in 1978 as having CGL when she presented with a WBC of 300×10^9/liter. Her spleen extended 11 cm below the left costal margin. The differential WBC was typical of chronic-phase CGL, and the leukocyte alkaline phosphatase score was 0. She was successfully treated with intermittent busulfan or hydroxyurea and remained well until July, 1983, when her disease underwent acute blastic transformation. A course of cytosine arabinoside was given without clinical benefit. The patient was then given 2 g of hydroxyurea daily. On admission, she had a WBC of 206×10^9/liter with 30% myeloblasts and 34% promyelocytes (Fig. 1). Her course was complicated by the development of fever, painful splenomegaly, thrombocytopenia, the adult respiratory distress syndrome, renal failure, hepatic insufficiency, and gastrointestinal bleeding. Leukapheresis was initiated to control the white count but was only transiently successful (Fig. 1).

In vitro differentiation. In vitro incubation of mononuclear cells following a 4-h exposure with mithramycin resulted in a rapid increase in the proportion of mature myeloid cells (Fig. 2). The number of viable cells varied <10% from base line. No evidence of differentiation was noted in the control cells incubated without mithramycin.

Clinical response. Before therapy, there was a rapid rise of the WBC despite continuous therapy with hydroxyurea. The WBC was only transiently controlled by leukapheresis (Fig. 1). The patient was then treated on a regimen of mithramycin of 1,225 μg i.v. every other day. Hydroxyurea, 2 g by mouth/d, was continued. The total white count remained relatively constant for 9 d and then fell precipitously. The absolute blast count began to fall after 6 d of treatment. A transient marked increase in myelocytes and metamyelocytes appeared between days 7 and 10 (Fig. 3). By day 7 of therapy, the patient became afebrile, was weaned off oxygen, and had a striking clinical and biochemical improvement that continued for 3 wk. She received a total of eight doses of mithramycin.

Inhibition of RNA synthesis. Whole blood RNA synthesis was measured serially (Fig. 4). There was a 40% inhibition of total cellular RNA synthesis by day 2 and an 80% inhibition of RNA synthesis by day six, 1 d before clinical evidence of differentiation occurred.

Expression of c-abl, c-myc, and lysozyme genes. We investigated the expression of the *c-myc* and *c-abl* oncogenes as well as the lysozyme gene in this patient's leukemic cells before and during mithramycin treatment. Increased expression of the lysozyme gene is a marker of increasing myeloid differentiation. Fig. 5 shows the concentration of *c-myc, c-abl,* and lysozyme transcriptions on days 0, 2, and 10 of therapy. Both *c-myc* and *c-abl* were expressed at very high levels in the untreated cells, whereas the lysozyme transcript was barely detectable. The day 2 sample, obtained before any decrease in WBC, decrease in blasts, or increase in mature granulocytes,

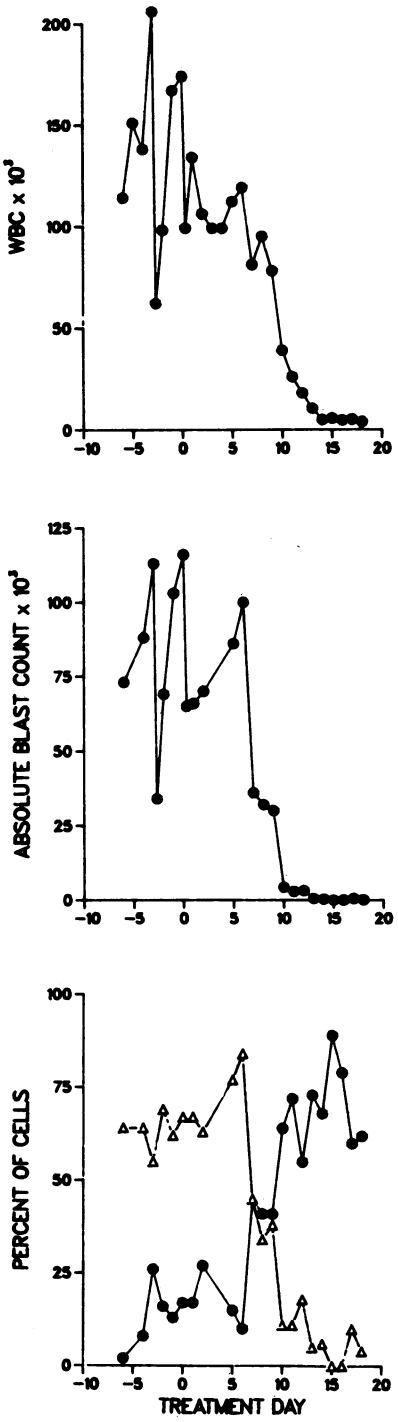

Figure 1. Hematologic parameters of the patient. *Top,* total WBC; *middle,* absolute blast count; *bottom,* percent blasts and promyelocytes (△) or PMN and bands (●). Leukapheresis performed on days −3 and 0. Day 0 is the first day of treatment with mithramycin.

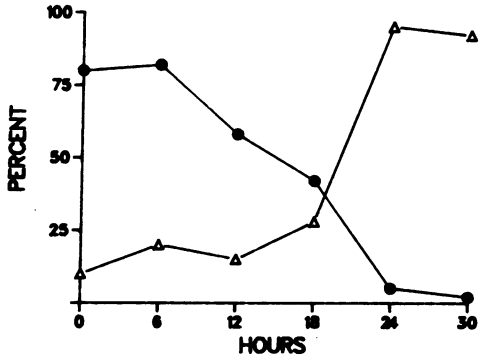

Figure 2. In vitro differentiation of pretreatment mononuclear cells exposed to 4.6×10^{-7} M mithramycin for 4 h. Percent blasts and promyelocytes (●), percent PMN, bands, and metamyelocytes (△). There was no decrease in the percent blasts and promyelocytes in the control flask.

demonstrated a >90% decrease in concentration of *c-myc* transcript and a >80% decrease in the *c-abl* transcript. Lysozyme gene expression increased significantly by day 2, indicating that mithramycin therapy did not cause universal inhibition of RNA synthesis. The changes noted by day 10, when evidence of a significant clinical response had occurred, are even more striking.

Discussion

Clinically, CGL usually develops in two phases. The initial phase, to which the name CGL applies most literally, is of variable duration and is marked by increased hematopoietic proliferative activity with normal differentiation (31). This chronic phase is usually superceded by a rapid fatal acute blastic transformation for which there is no known effective treatment (32). The blasts appear to be derived from a pleuripotential hematopoietic stem cell capable of self-renewal but incapable of maturation (33). Commonly, the patients die of infection due to a paucity of functional granulocytes.

A question of profound therapeutic importance is whether the leukemic blasts can be induced to mature under certain external conditions (3, 34). Work done with murine and human leukemic cell lines in vitro has indicated that some leukemic cells can be induced to differentiate into functional hematopoietic cells by a variety of agents, and in doing so lose their proliferative capability (35). In a murine model, induction of differentiation in vivo is associated with a significant increase in survival (4).

Although we could not completely rule out the possibility that mithramycin was specifically toxic to CGL blasts, several observations suggest that in vivo differentiation may have occurred in this patient. We showed that mithramycin caused this patient's leukemic cells to differentiate in vitro into normal appearing granulocytes, an observation that could not be accounted for by selective toxicity to immature cells. Furthermore, after initiation of therapy, we observed a substantial increase in the number of mature myelocytes and metamyelocytes before a decrease in the total WBC. The changes in morphology and number of circulating leukemic cells in this patient are temporally consistent with those observed in the mouse M-1 leukemia model treated successfully with differentiating agents (4).

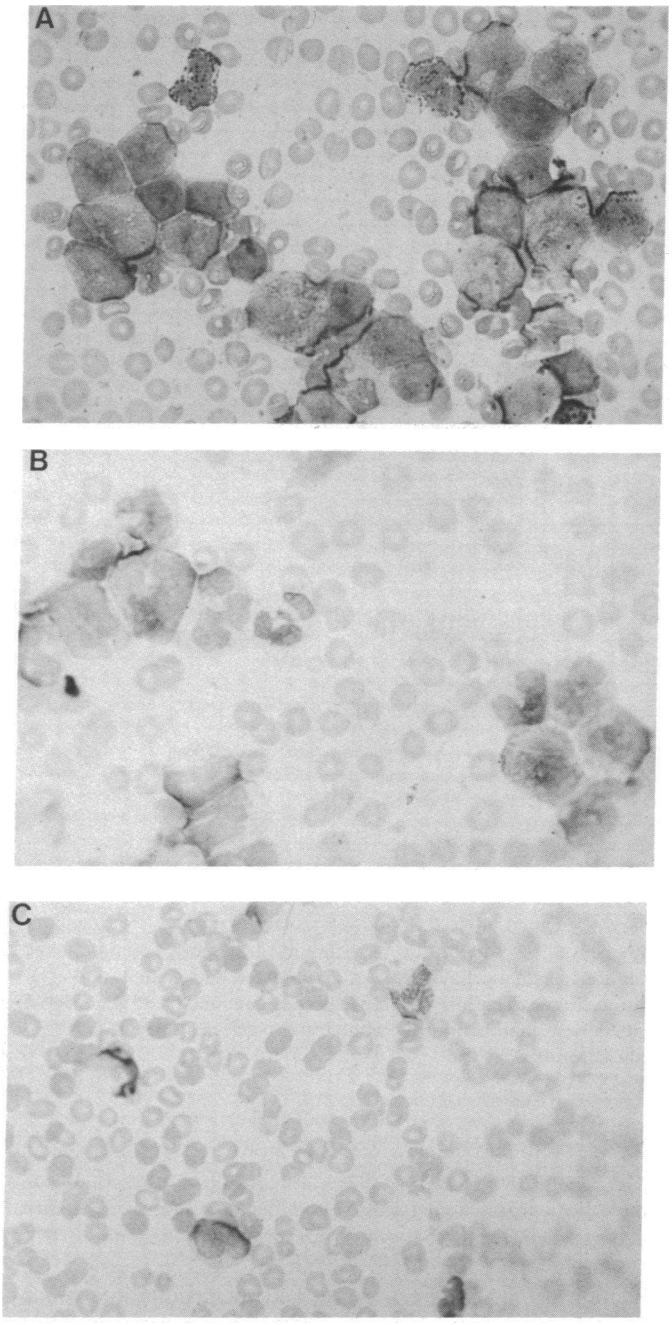

Figure 3. Photomicrographs of peripheral blood films. (*A*), Day 0; (*B*), day 8; and (*C*), day 12.

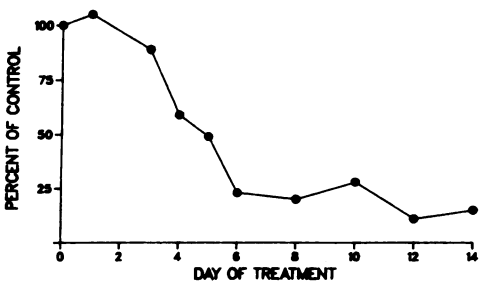

Figure 4. Whole blood RNA synthesis during mithramycin therapy. RNA synthesis was measured as described in text.

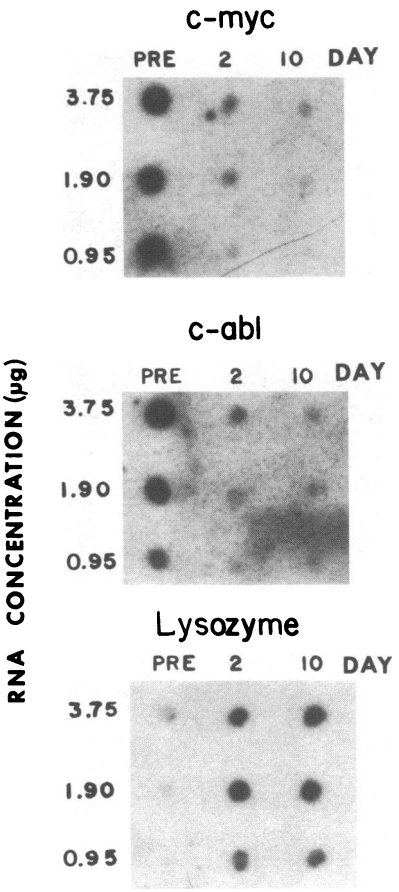

Figure 5. Quantitative RNA dot-blot analyses of RNA from leukemia cells of the patient during mithramycin therapy. Serial doubling dilutions of total cellular RNA were prepared and blotted onto nitrocellulose. The first dot in each series contained 3.75 μg of RNA. Filters were then hybridized with *c-myc, c-abl,* or lysozyme ^{32}P-labeled probes.

We were particularly interested in expression of *c-myc* and *c-abl* protooncogenes, in this case because these oncogenes have been implicated in CGL and because the mechanism of activity of mithramycin is inhibition of DNA-dependent RNA synthesis; that is, mithramycin inhibits gene expression. Correlation of oncogene expression and therapeutic intervention has not yet been reported in humans, although *c-myc* expression decreases dramatically in HL60 cells induced to differentiate in vitro with vitamin D (36). Thus, we were interested to observe that the levels of expression of both the *c-myc* and *c-abl* protooncogenes in the leukemic cells of our patient decreased dramatically after initiation of mithramycin therapy. These major changes in oncogene expression were observed before any morphological changes occurred.

The association of markedly decreased *c-myc* and *c-abl* oncogene expression with evidence for in vivo differentiation of these cells may have simply reflected the altered proliferative state of these cells after treatment with an effective agent (22). However, a substantial decrease in *c-myc* and *c-abl* expression was noted before decreases in either the absolute leukemic cell number or the relative proportion of leukemic blasts to normal cells. Furthermore, although both total cellular and oncogene expression was inhibited by mithramycin, expression of lysozyme gene, a marker of increasing differentiation, was augmented. These data suggest that mithramycin may have allowed differentiation to occur by inhibiting expression of specific gene products that prevent differentiation. The data also imply that there is a therapeutic index for mithramycin in regard to selective inhibition of RNA synthesis.

The ability of mithramycin to induce differentiation both in vitro and possibly in vivo suggests that it may have a role in conjunction with cytotoxic agents in the therapy of certain patients with blast-phase CGL. It is likely that alterations in either the structure or expression of several oncogenes is required in the multistep progression to cancer (37). Under some circumstances, inhibition of abnormally increased expression may be associated with beneficial clinical responses. The fact that this patient showed considerable clinical improvement during the time she responded to this treatment is encouraging. Mithramycin appears to be the first agent to have shown evidence of in vivo differentiation in the blast phase of CGL.

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