COMPLETE REMISSION AFTER TREATMENT OF ACUTE PROMYELOCYTIC LEUKEMIA WITH ARSENIC TRIOXIDE

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ABSTRACT

Background. Two reports from China have suggested that arsenic trioxide can induce complete remissions in patients with acute promyelocytic leukemia (APL). We evaluated this drug in patients with APL in an attempt to elucidate its mechanism of action.

Methods. Twelve patients with APL who had relapsed after extensive prior therapy were treated with arsenic trioxide at doses ranging from 0.06 to 0.2 mg per kilogram of body weight per day until visible leukemic cells were eliminated from the bone marrow. Bone marrow mononuclear cells were serially monitored by flow cytometry for immunophenotype, fluorescence in situ hybridization, reverse-transcription–polymerase-chain-reaction (RT-PCR) assay for PML–RARα fusion transcripts, and Western blot analysis for the expression of the apoptosis-associated proteins caspases 1, 2, and 3.

Results. Of the 12 patients studied, 11 had a complete remission after treatment that lasted from 12 to 39 days (range of cumulative doses, 160 to 515 mg). Adverse effects were relatively mild and included rash, lightheadedness, fatigue, and musculoskeletal pain. Cells that expressed both CD11b and CD33 (antigens characteristic of mature and immature cells, respectively), and which were found by fluorescence in situ hybridization to carry the t(15;17) translocation, increased progressively in number during treatment and persisted in the early phase of complete remission. Eight of 11 patients who initially tested positive for the PML–RARα fusion transcript by the RT-PCR assay later tested negative; 3 other patients, who persistently tested positive, relapsed early. Arsenic trioxide induced the expression of the proenzymes of caspase 2 and caspase 3 and activation of both caspase 1 and caspase 3.

Conclusions. Low doses of arsenic trioxide can induce complete remissions in patients with APL who have relapsed. The clinical response is associated with incomplete cytodifferentiation and the induction of apoptosis with caspase activation in leukemic cells. (N Engl J Med 1998;339:1341–8.)

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ACUTE promyelocytic leukemia (APL) represents 10 to 15 percent of the cases of acute myeloid leukemia in adults. This disease is characterized by a specific cytogenetic abnormality — reciprocal chromosomal translocations that disrupt the retinoic acid receptor α gene (RARα) on chromosome 17 and the promyelocytic leukemia gene (PML), which encodes a transcription factor and is located on chromosome 15. The resulting fusion gene, PML–RARα, encodes a chimeric protein that causes an arrest of maturation at the promyelocyte stage of myeloid-cell development.

Recent advances have dramatically improved the outcome of treatment of this disease. Since 1990, the incorporation of all-trans-retinoic acid into chemotherapy has more than doubled the survival expected with chemotherapy alone. The risk of relapse in patients who initially achieve remission with modern therapy has decreased to approximately 20 percent, as compared with 60 to 70 percent in the 1980s. Moreover, all-trans-retinoic acid provided the first proof of the principle of "differentiation therapy," in which drugs induce the terminal differentiation of malignant cells that are then incapable of further proliferation. Despite earlier optimism, this concept has not yet been usefully extended to other cancers.

Recently, investigators from China reported that arsenic trioxide could induce complete remissions in patients with APL. Preclinical studies suggested that this agent induced apoptosis; however, one
study suggested that leukemic cells differentiated after prolonged exposure to the drug. We initiated a study to evaluate this agent in patients with APL who had relapsed despite having received the best current therapy. Our results show that low doses of arsenic trioxide are strikingly effective and cause few serious adverse reactions. The clinical response to arsenic trioxide is associated with the induction of "nonterminal" cytodifferentiation and the activation of cytotoxic proteases (caspasas) that are characteristic of apoptosis.

METHODS

Clinical Protocol

The eligibility criteria of our study included a diagnosis of APL confirmed by cytogenetic analysis or fluorescence in situ hybridization for patients with a t(15;17) translocation, or by the reverse-transcription-polymerase-chain-reaction (RT-PCR) assay for PML-RARα fusion transcripts. In patients who had relapsed after standard therapy that included all-trans-retinoic acid plus a combination of cytotoxic drugs. Written informed consent was required, and the protocol was reviewed and approved by the institutional review board of the Memorial Sloan-Kettering Cancer Center.

Treatment with Arsenic Trioxide

Arsenic trioxide was supplied as an aqueous solution in 10-ml vials containing 1 mg of drug per milliliter. The drug was further diluted to 500 ml of 5 percent dextrose solution and infused intravenously over a period of two to four hours once per day. The initial cohort of patients received either 10 or 15 mg of arsenic trioxide per day as a fixed dose, but the referral of two children to the study prompted conversion to a weight-adjusted regimen (0.15 mg per kilogram of body weight per day). The drug was given daily until visible leukemic blasts and promyelocytes were eliminated from the bone marrow and the residual blast count was no more than 5 percent of marrow mononuclear cells. Patients who had complete remission were eligible for treatment with additional courses of therapy three to six weeks after the preceding course. Subsequent courses were generally given at a dose of 0.15 mg per kilogram per day for a cumulative total of 25 days; the drug was administered either daily or on a weekend-only schedule, for a maximal total of six courses over a period of approximately 10 months.

Monitoring

Patients with coagulopathy received transfusions of platelets and fresh frozen plasma to maintain the platelet count and fibrinogen at target levels of at least 50,000 cells per cubic millimeter and 100 mg per deciliter, respectively. Blood counts, coagulation studies, serum chemistry profiles, urinalyses, and electrocardiograms were performed serially. Bone marrow aspiration or biopsy (or both) was performed at base line and periodically thereafter until remission was documented. Conventional criteria for a response were used, including no more than 5 percent blasts in bone marrow, a peripheral-blood leucocyte count of at least 3000 cells per cubic millimeter, and a platelet count of at least 100,000 cells per cubic millimeter.

Cellular Immunophenotyping Studies

Bone marrow or blood samples were treated with heparin, and mononuclear cells were isolated by Ficoll-Hypaque centrifugation. Surface-membrane antigens were detected by direct immunofluorescence staining with the use of fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies: CD16 (Leu-11a), CD11b, CD33 (Leu-M9), HLA-DR, CD45, and CD14 (Becton Dickinson, Mountain View, Calif.; and Immunotech Immunology, Marseilles, France). Dual-color staining was performed by incubating cells simultaneously with two monoclonal antibodies, including CD33–PE and CD11b–FITC or CD33–PE and CD16–FITC. Negative controls in which irrelevant monoclonal immunoglobulins of the same isotype were used were analyzed concurrently. Flow-cytometric analyses were performed on an Epics Profile II flow cytometer ( Coulter Electronics, Hialeah, Fla.) equipped with a 488-nm argon laser. Measurement of forward and side scatter was combined with CD45–CD14 staining to identify cell populations of interest and to exclude monocytes. The Multiparameter Data Acquisition and Display System (MDADS, Coulter Electronics) was used to acquire and analyze data.

Fluorescence in Situ Hybridization

Selected specimens that had undergone immunofluorescence staining for CD33 and CD11b were sorted to identify cells that expressed both antigens with a FACStar Plus cell sorter (Becton Dickinson). Separated cells were incubated in culture medium at 37°C for one hour, treated with a hypotonic solution of potassium chloride (0.075 M) for five minutes, fixed in a 3:1 methanol–acetic acid solution, and air-dried. Interphase fluorescence in situ hybridization was performed with the use of biotinylated RARα translocation dual-color probe (Dysis, Downer's Grove, Ill.). Briefly, DNA from cells in interphase was denatured by immersing slides in a solution of 50 percent formamide and 2x saline sodium citrate (SSC) buffer (1x SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) at 73°C for five minutes; the slides were then dehydrated in alcohol and air-dried. A probe in hybridization mixture was placed, allowed to a glass slide under a coverslip, and sealed with rubber cement. Hybridization was carried out at 37°C in a moist chamber for approximately 12 to 16 hours. After hybridization, unbound probe was removed by washing slides at 45°C in 50 percent formamide and 2x SSC three times for 10 minutes each, followed by washing in 2x SSC and 0.1 NP-40 solution at 45°C for 5 minutes. Slides were then air-dried and counterstained with 4',6-diamidino-2-phenylindole and placed under a glass coverslip. Cells in interphase were analyzed for fluorescence signals with a Photometrics SenSys camera (Photometrics, Tucson, Ariz.) fitted to a Zeiss axiophot microscope (Zeiss, Thornwood, N.Y.). A minimum of 300 cells was studied for each sample.

Western Blot Analysis

Cells were lysed in a buffer containing 50 mM TRIS–hydrochloric acid, 0.5 M dithiothreitol (Sigma)-thiourea (additive), 170 mM sodium chloride, 1 mM dithiothreitol, 0.2 percent NP-40, 0.01 U of aprotinin per milliliter, 10 μg of leupeptin per milliliter, 10 μg of pepstatin per milliliter, and 1 μM phenylmethylsulfonyl fluoride (all from Sigma, St. Louis). The lysates were then sonicated with an ultrasonic homogenizer (4710 series, Cole Parmer, Chicago) and centrifuged at 7500 x g (Sorvall, Newtown, Conn.). The protein content of the lysates was determined with a BioRad protein assay kit (BioRad Laboratories, Hercules, Calif.) at 595 nm with bovine serum albumin used as the standard. A sample buffer containing 10 percent glycerol, 0.4 percent sodium dodecyl sulfate (SDS), 0.3 percent bromphenol blue, and 0.2 percent pyronin Y in 1x stacking buffer (0.5 M TRIS base and 0.8 percent SDS) and 20 percent 2-mercaptoethanol was added to the cell lysates, which were heated-denatured at 95°C for three minutes. Subsequently, 15 μg of protein per lane was loaded on a SDS–polyacrylamide gel containing 12.5 percent polyacrylamide and was size-fractionated by electrophoresis. Proteins were electroblotted onto Trans-Blot transfer medium (Bio-Rad) and stained with Ponceau-S to serve as an internal loading control. Rabbit antihuman monoclonal antibodies against caspase 1, caspase 2 (both from Santa Cruz Biotechnology, Santa Cruz, Calif.), and caspase 3 (PharMingen, San Diego, Calif.) were added, and bound antibodies were detected with the ECL chemiluminescence system (Amer sham, Arlinglon Heights, Ill.). The protein bands were quantified by computer densitometry.
TABLE 1. CLINICAL CHARACTERISTICS OF THE PATIENTS AND RESULTS OF THERAPY WITH ARSENIC TRIOXIDE.*

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<th>PATIENT NO.</th>
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<th>CUMULATIVE DOSE (mg)</th>
<th>TIME TO REMISSION (days)</th>
<th>TIME TO PLATELET COUNT &gt;100,000/mm³ (days)</th>
<th>TIME TO LEUKOCYTE COUNT &gt;3000/mm³ (days)</th>
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*All the patients had previously received one or more courses of all-trans-retinoic acid, plus an anthracycline antibiotic and cytarabine.
†The patient had proved retinoid resistance (i.e., a lack of response to treatment during reinduction or a relapse while receiving retinoid maintenance therapy).
‡The patient had also received mitoxantrone and etoposide.
§The patient also underwent allogeneic bone marrow transplantation.
‖The patient also received 9-cis-retinoic acid plus M195 (a monoclonal antibody to CD43).
*The patient had also received mercaptopurine, vincristine, and mercaptopurine.
**The patient died on day 5 of the study.

RT-PCR Analysis for PML–RAR-α Fusion Transcripts

RT-PCR was performed according to previously described methods.19,20

RESULTS

Patients

Twelve patients with relapsed APL were treated. All the patients had received extensive prior therapy with retinoids and cytotoxic drugs (Table 1). Two patients had relapsed after allogeneic bone marrow transplantation; one of these patients also had no a response to reinduction with donor T cells. One patient was undergoing hemodialysis for chronic renal failure.

Clinical Efficacy

Eleven of the 12 patients had a complete remission after treatment with arsenic trioxide. The one patient who entered the trial while on hemodialysis sustained an intracranial hemorrhage on day 1 and died on day 5. The median duration of therapy in the 11 patients who responded to treatment was 33 days (range, 12 to 39), the median daily dose was 0.16 mg per kilogram (range, 0.06 to 0.20), and the median cumulative dose during induction was 360 mg (range, 160 to 515) (Table 1). Complete remission according to all criteria was attained by a median of 47 days (range, 24 to 83) after the initiation of therapy. Remission according to bone marrow criteria — the determining factor for discontinuing therapy — was achieved first and was usually followed by the recovery of peripheral-blood leukocytes and then by the recovery of platelets. With respect to the range of doses used in this study, no differences in efficacy or time to response were obvious. After two courses of therapy, 8 of 11 patients tested negative for the PML–RAR-α fusion transcript by RT-PCR assay.

All 11 of the patients in complete remission completed at least one course of treatment with arsenic trioxide after remission. Four patients completed three courses, two completed four courses, and one completed five courses. The median duration of remission was more than five months (range, one to more than nine). However, 3 of the 11 patients relapsed during the second course of treatment; in none of these patients had the RT-PCR result converted to negative, and each patient appeared to have acquired drug resistance rapidly. Two of these patients have since died of progressive leukemia.

Adverse Events

The clinical condition of the patients in this study was highly variable, which reflected the extensive prior therapy they had received. The protocol did not require hospitalization; three patients completed induction therapy entirely as outpatients, and one oth-
Figure 1. Expression of Surface Antigens from Bone Marrow Mononuclear Cells during Therapy with Arsenic Trioxide (Upper Panel) and the Effect of Therapy on the Immunophenotype (Lower Panels).

CD33 is an antigen usually found on immature myeloid and leukemic cells, whereas CD11b is found on mature cells, including granulocytes. When the flow cytometer is adjusted to select cells that simultaneously express both CD33 and CD11b, a unique population of cells is detected. These dual-expressing cells unexpectedly persisted for extended periods after the achievement of complete remission (upper panel). The curves with the open symbols indicate the proportions of cells that expressed CD33 only. The curves with the solid symbols and the shaded region denote cells that simultaneously express both CD33 and CD11b. (Data from four patients are shown.)

The effect of arsenic trioxide on the immunophenotype of bone marrow mononuclear cells from one patient is shown in a dual-variable scatterplot in the lower panels. Before treatment, the majority of cells expressed only CD33, a pattern typical of APL (left-hand plot). After 15 days of therapy, approximately 80 percent of the CD33 cell population had been induced to express CD11b, an antigen characteristic of late myeloid differentiation (middle plot). Continued therapy further shifted the cell populations, and a large majority of cells simultaneously coexpressed CD33 and CD11b (right-hand plot). The horizontal and vertical lines in the scatterplots represent window settings for quantitative discrimination between variables.
er patient was hospitalized solely for the placement of a venous catheter. However, eight patients were hospitalized for complications of relapsed leukemia; five of these eight patients required transfer to an intensive care unit, endotracheal intubation, and assisted ventilation for complications that included pulmonary hemorrhage, renal failure, sepsis, graft-versus-host disease, nonspecific pulmonary infiltrates, and hypotension. One patient required the insertion of a permanent pacemaker after second-degree heart block developed in the setting of severe metabolic acidosis, hyperkalemia, hypotension, and renal insufficiency. However, the heart block reversed despite rechallenge with further arsenic trioxide therapy.

Administration of the drug was temporarily suspended in five patients for a median of two days (range, one to five) because of serious intercurrent medical complications. In two patients, symptoms similar to those of the “retinoic acid syndrome” developed; both patients were treated with dexamethasone, and their symptoms improved. Only two patients required no platelet transfusions; the median number of units of platelets transfused was 61 (range, 0 to 586).

The median total peripheral-blood leukocyte count at the time of study entry was 4700 cells per cubic millimeter (range, 500 to 144,000). In six patients, leukocytosis (i.e., a leukocyte count of ≥20,000 per cubic millimeter) developed (range, 20,800 to 144,200). No additional therapy was administered to these patients, and the leukocytosis resolved in all cases without further intervention.

Common adverse reactions included lightheadedness during the infusion, fatigue, musculoskeletal pain, and mild hyperglycemia. Three patients had dysesthesias, presumably due to peripheral neuropathy. However, two of these patients had been immobilized for prolonged periods during assisted ventilation, and the other patient had a history of neuropathy.

Immunophenotyping Studies

In APL, the leukemia cells express CD33, an antigen typically associated with primitive myeloid cells. Therapy with arsenic trioxide induced a progressive decrease in the proportion of cells that expressed CD33, along with an increase in the proportion of cells that expressed CD11b, an antigen associated with mature myeloid elements. These changes would be anticipated after therapy with any agent that induced remission of APL, but arsenic trioxide also induced the expression of cells that simultaneously expressed both antigens (Fig. 1). In most cases, these cells dominated the myeloid-cell population, and they persisted for extended periods after complete remission was achieved according to clinical criteria. Figure 1 (lower panels) shows serial scatter displays of bone marrow mononuclear cells from one patient.

Fluorescence in Situ Hybridization Analysis

Bone marrow mononuclear cells taken from a patient during both early and later phases of complete remission were sorted by flow cytometry to measure the coexpression of CD33 and CD11b. We used fluorescence in situ hybridization analysis to examine
cells early in remission. In a fashion similar to that of control APL cells (Fig. 2A), the majority of these cells yielded a hybrid signal, indicating a translocation between the PML and RAR-α genes (Fig. 2B) and their origin from the neoplastic clone. However, when cells from the same patient were sorted according to the same variables later in remission, only the normal pattern of fluorescence signals was detected (Fig. 2C), indicating that these cells had been derived from normal hematopoietic progenitors.

Western Blot Analysis

Protein extracts taken from bone marrow mononuclear cells were serially examined by Western blot analysis. As shown in Figure 3, the precursor forms of caspase 2 and caspase 3 were up-regulated in one patient in response to treatment with arsenic trioxide. Moreover, this treatment also induced the expression of cleaved fragments of caspase 1, indicating activation of the enzyme. Additional data also indicated increased expression of the cleaved form of caspase 3 (data not shown). (The antibody used in these experiments does not react with the cleaved form of caspase 2.)

DISCUSSION

In this study, we confirmed preliminary reports of the striking activity of arsenic trioxide against APL, initially noted by investigators from Harbin and Shanghai, China. With few exceptions, the patients in our trial had multiple relapses and had disease that was resistant to conventional chemotherapy, retinoids, or bone marrow transplantation. At the time of study entry, the patients had numerous complications related to relapsed leukemia, including respiratory failure, disseminated varicella-zoster infection, cavitary aspergillosis, chronic renal failure, and graft-versus-host disease. Moreover, 5 of the 12 patients required admission to an intensive care unit for assisted ventilation and supportive care, but these complications were not directly related to therapy with arsenic trioxide.

Without randomized studies, comparisons of arsenic trioxide with other therapies are premature. Nonetheless, it is intriguing that we and the Shanghai investigators—both groups with early experience using all-trans-retinoic acid—both found that virtually all patients with a confirmed diagnosis of APL attained remission after treatment with arsenic trioxide without the early mortality associated with retinoid therapy. Although less commonly observed than with all-trans-retinoic acid treatment, striking leukocytosis was induced by arsenic trioxide in several patients. We elected to withhold other cytotoxic drugs, and the leukocytosis disappeared as patients entered remission. Although there were 3 early relapses, the RT-PCR assays for the PML-RAR-α fusion transcript (a molecular marker of residual disease) showed that 8 of the 11 patients who initially tested positive later tested negative, a phenomenon that is unusual after treatment with all-trans retinoic acid alone.

Although quite preliminary, our data suggest that arsenic trioxide is active in APL at doses ranging
from 0.06 to 0.20 mg per kilogram. Within this range, no relation between dose and efficacy was obvious; however, the patient treated with the highest dose had a characteristic skin reaction to arsenic. These findings are especially important, since severe toxic reactions, including flaccid paralysis and renal failure, have been observed after attempts to increase the dose beyond this range.27

The mechanisms of action of arsenic trioxide in this disease are being actively studied, but some preliminary observations are pertinent. In vitro, arsenic was shown to reorganize a nuclear organelle17 (known as the “PML oncogenic domain”) that is disrupted in patients with APL.28 and also to degrade the mutant PML–RAR-α fusion protein15,12,28 formed as a result of the (t(15;17)) translocation. These observations might argue that the activity of arsenic could by definition be restricted to APL. However, using embryonic fibroblasts from mice in which the PML gene was inactivated by homologous recombination, we recently showed that the activity of arsenic in myeloid cell lines was independent of both PML and PML–RAR-α.29 Furthermore, we30,31 have shown that the agent has broad activity against a variety of both hematologic and solid-tumor cell lines in vitro. These results are notable, since arsenicals have been used as medicines for thousands of years,32 in particular for chronic myelocytic leukemia until the 1930s33 and currently for African sleeping sickness due to infection with Trypanosoma brucei.34

All-trans-retinoic acid induces terminal differentiation of APL cells,9–11 but the cytodifferentiating effects of arsenic trioxide appear to be incomplete. Arsenic induces a population of cells that coexpress surface antigens characteristic of both mature and immature cells (i.e., CD11b and CD33).35 Early during induction, these cells retain the (t(15;17)) translocation that characterizes APL. Unexpectedly, these cells persisted in the bone marrow of the patients in our study despite a clinically complete remission; however, later in remission, the cells coexpressing these two antigens — although still readily detectable — were no longer positive for the translocation when analyzed by in situ hybridization. The morphologic appearance of leukemic cells during therapy with arsenic trioxide was also far less distinctive than that observed during therapy with all-trans-retinoic acid.11 In fact, leukemic cells from many patients had few morphologic changes for 10 or more days, after which the proportion of leukemic cells progressively decreased.

After nonterminal differentiation, arsenic trioxide appeared to induce apoptosis, coincident with the increased expression of cysteine proteases (caspases) and their conversion from inactive precursors to activated enzymes. The caspase pathway has only recently been characterized as an important pathway of programmed cell death. Initially recognized be
cause of the homology between the Caenorhabditis elegans protein CED-3 and mammalian interleukin-1β-converting enzyme,36 the family of caspases now encompasses at least 10 proteins that cleave a number of polypeptides.37,38 In leukemic cell lines, caspase activation can be induced by a number of cytotoxic agents,39 including all-trans-retinoic acid.40,41 Since these enzymes induce widespread proteolysis, it is conceivable that the PML–RAR-α transcript is a caspase substrate.

Another feature shared by arsenic trioxide and all-trans-retinoic acid is the rapid development of clinical resistance in some patients. Leukemic cells taken from two patients who relapsed retained sensitivity to arsenic in culture at concentrations ranging from 10–4 M to 10–7 M (unpublished data). Relative arsenic resistance due to decreased intracellular transport has been described in association with the down-regulation of membrane transporters encoded by the atr operon in bacterial cells.42 Resistance in mammalian cells is less well characterized, but alterations in membrane transport or efflux are probably important factors.43

In summary, arsenic trioxide can induce a complete remission in patients with APL who have relapsed after extensive prior therapy. This drug causes partial but incomplete cytodifferentiation of leukemic cells, followed by caspase activation and induction of apoptosis. The striking degree of activity of arsenicals in this disease, plus their lack of specificity for APL-specific proteins, suggests that they may warrant further study as therapy for other neoplastic diseases.

References

3. Bottom J, Goddard AD, Skeer D, Solomon E. Molecular analysis of


