

Retinoic Acid and Arsenic Synergize to Eradicate Leukemic Cells in a Mouse Model of Acute Promyelocytic Leukemia

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Summary

In acute promyelocytic leukemia (APL) patients, retinoic acid (RA) triggers differentiation while arsenic trioxide (arsenic) induces both a partial differentiation and apoptosis. Although their mechanisms of action are believed to be distinct, these two drugs both induce the catabolism of the oncogenic promyelocytic leukemia (PML)/RAR α fusion protein. While APL cell lines resistant to one agent are sensitive to the other, the benefit of combining RA and arsenic in cell culture is controversial, and thus far, no data are available in patients. Using syngenic grafts of leukemic blasts from PML/RAR α transgenic mice as a model for APL, we demonstrate that arsenic induces apoptosis and modest differentiation, and prolongs mouse survival. Furthermore, combining arsenic with RA accelerates tumor regression through enhanced differentiation and apoptosis. Although RA or arsenic alone only prolongs survival two- to threefold, associating the two drugs leads to tumor clearance after a 9-mo relapse-free period. These studies establishing RA/arsenic synergy *in vivo* prompt the use of combined arsenic/RA treatments in APL patients and exemplify how mouse models of human leukemia can be used to design or optimize therapies.

Key words: differentiation • apoptosis • cancer • clinical trials • transgenics

Acute promyelocytic leukemia (APL)¹ is specifically associated with a t(15;17) translocation which generates a PML/RAR α fusion between the gene of a nuclear protein, promyelocytic leukemia (PML), and that of a transcription factor, the retinoic acid receptor α (RAR α). RA and RAR α are believed to contribute to myeloid differentiation (1, 2). PML, through its association with nuclear matrix domains of unknown function (PML nuclear bodies, NBs [3]), was shown to suppress growth (4–6) and to induce apoptosis (7–9). Some PML/RAR α transgenic mice develop a disease that strikingly resembles APL, establishing that PML/RAR α can initiate the leukemic process (10). PML/RAR α was shown to block myeloid differentiation (11), most likely through the impairment of RA response. The latter appears to result from the tighter binding to PML/RAR α compared with RAR α of corepressor proteins involved in transcriptional silencing (12, 13). Con-

versely, PML/RAR α also delocalizes PML from NBs (14–17) and blocks apoptosis (11, 18, 19). Hence, in this model, PML/RAR α exerts a double dominant-negative effect on the function of both RAR α and PML proteins (20, 21).

RA and arsenic trioxide (arsenic) were shown to be clinically effective in APL treatment through the induction of differentiation and apoptosis, respectively (22, 23). In non-APL cells, RA binds to RARs, activating transcription of target genes, whereas arsenic alters the traffic of PML proteins, enhancing their NB association as well as their apoptotic properties (7, 24, 25). In addition, in APL cells, both drugs degrade PML/RAR α (24–27). It is not yet clear which of the actions of these two drugs, on the fusion or on the normal RAR α or PML alleles, is responsible for their distinct biological effects (differentiation versus apoptosis). These drugs would be expected to synergize, since cell lines resistant to one agent remain sensitive to the other (28–30). However, current evidence obtained *in vitro* is conflicting (28–30). In this report, we have established an *in vivo* APL model by transplanting leukemic blasts from PML/RAR α transgenic mice. Although arsenic or RA only modestly prolongs survival, combining the two

¹Abbreviations used in this paper: APL, acute promyelocytic leukemia; arsenic, arsenic trioxide; NB, nuclear body; PML, promyelocytic leukemia; RA, retinoic acid; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

agents induces faster tumor regression and sharply prolongs survival. These studies exemplify how mouse models of human leukemia can be used to optimize therapies and prompt the use of combined arsenic/RA treatments in APL patients.

Materials and Methods

Transplantation of Leukemia and Arsenic/RA Treatments. Leukemic cells were isolated from bone marrow and spleen of leukemic hMRP8-PML/RAR α transgenic mice (leukemia 935) as described (10), by flushing RPMI medium through long bones and collecting exudates from spleen. In vitro, spleen cells were cultured in RPMI medium supplemented with 10% FCS and 2% pokeweed mitogen spleen-conditioned medium and were left untreated or were treated with 1 μ M RA, 1 μ M As₂O₃ (Sigma Chemical Co.), or both.

Leukemias were propagated by injecting blasts (10⁷ viable hematopoietic cells) into the tail vein of 6–7-wk-old syngenic FVB-NICO mice. Animal handling was done according to the guidelines of institutional animal care committees. Mice implanted with leukemic cells were randomly assigned to either type of treatment. RA was administered to leukemic mice by subcutaneous implantation of a 21-d release pellet containing 10 mg ATRA (Innovative Research of America). A stock solution of 330 mM As₂O₃ was prepared by diluting the powder in 1 M NaOH, then a dilution in Tris-buffered saline (TBS) was administered by daily intraperitoneal injection at the concentration of 5 μ g/g mice. Control mice were treated with placebo pellets or intraperitoneal injections of TBS.

Histological and Cytological Analyses. Specimens of spleen, liver, and lung were cut into three parts and immediately processed for snap freezing in liquid nitrogen or fixations. Specimens of long bones were fixed in formaldehyde, decalcified in 10% nitric acid, and further processed for paraffin embedding. Spleen, liver, and lung were either fixed in alcohol-formaldehyde-acetic acid reagent (AFA; Carlo Erba Laboratories), paraffin embedded and stained with hematoxylin-eosin and May-Grünwald-Giemsa, or fixed in 2.5% glutaraldehyde in cacodylate buffer and epon embedding for electron microscopic examination. The extent of the leukemic infiltrate was assessed on paraffin sections. The differentiation of the leukemic cells was assessed by combining cytological and histological stains, immunofluorescent staining of cryocut sections with a rat anti-mouse CD11b antibody (PharMingen) and electron microscopic analysis. In situ cell death was studied by morphological analysis on paraffin sections, electron microscopic grids, and by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays (reagents from Boehringer Mannheim), both on paraffin and cryocut sections.

Results

Leukemic Cells from PML/RAR α Transgenic Mice Are Arsenic Sensitive. hMRP8-PML/RAR α transgenic mice develop transplantable leukemias which differentiate both in vivo and in vitro upon RA exposure (10). To test their sensitivity to arsenic in vitro, leukemic cells were isolated from spleen or bone marrow of moribund animals and cultured in the presence or absence of arsenic. Little apoptosis and no differentiation were observed by TUNEL or cytological examinations. Conceivably, growth factors present in conditioned media may block apoptosis, as demonstrated

in other cellular settings. However, both arsenic and RA induced PML/RAR α degradation (data not shown), as shown previously in APL cell lines (25, 26), confirming that degradation of the fusion protein does not suffice to trigger arsenic-induced apoptosis (30).

Syngenic FVB mice were then injected with 10⁷ leukemic cells. Transplantation was always successful, as all animals died with an intraexperimental variation of <1 wk, generally in 30–50 d. In dose-response experiments, mice were treated for 1 mo with daily injections of arsenic or TBS 4 d after leukemia engraftment. Although 1 μ g/g body wt arsenic daily yielded no tumor regression upon killing, 10 μ g/g led to many early deaths, presumably of toxic origin (pathological examination revealed some hepatic toxicity and widespread pulmonary edema). However, with 5 μ g/g, arsenic-treated animals showed greatly reduced leukemic infiltrate of the organs analyzed. As nontransplanted mice treated with this same dose for the same length of time also showed no evidence for toxicity, a daily dose of 5 μ g/g was used thereafter. Despite the much higher doses used in mice compared with humans, the circulating arsenic levels were in the range of those present in arsenic-treated APL patients (31; data not shown). In pilot survival experiments where mice were treated 4 d after transplantation for 38 d, the 10 arsenic-treated mice lived significantly longer than the 10 controls (mean: 124 \pm 6 vs. 50 \pm 4 d). Altogether, our results demonstrate that leukemic cells from PML/RAR α transgenic mice are arsenic sensitive in vivo.

RA and Arsenic Synergize to Induce Tumor Regression. We have previously shown in cell lines that arsenic and RA appear to synergize for both differentiation and apoptosis (30), although this has been disputed (22, 28). To test the possible synergy between these two agents in vivo, we evaluated their effects on the regression of established leukemias. Hence, for this set of experiments, leukemias were allowed to develop for 20–25 d before therapy. Leukemic mice were then randomly assigned to treatment with arsenic, RA, both, or vehicle for 4 or 8 d and killed (two mice per treatment and time point). In three different experiments, RA or arsenic treatments reduced spleen weight and liver infiltration, whereas their association completely normalized the macroscopic appearance of these organs (not shown).

Microscopic examination of hematoxylin-eosin-stained sections of bone marrow, spleen, and liver from these animals confirmed this observation. In the absence of therapy, massive leukemic infiltration was evident in all three organs. In particular, the bone marrow was strictly monomorphic, consisting of promyelocyte-like cells that retained immature features such as basophilic cytoplasm (Fig. 1 A).

As reported previously, RA caused the rapid differentiation of leukemic cells into polymorphonuclear leukocytes. In the bone marrow, 4 d of RA treatment induced a drastic reduction of the cellular density with reappearance of some adipocytes (Fig. 1 B, and data not shown). Nevertheless, the marrow remained monomorphic, almost exclusively composed of polymorphonuclear cells (arrowheads, Fig. 1 B). After 8 d of RA, normal hematopoiesis was restored, with a large number of erythroblasts and a decrease in gran-

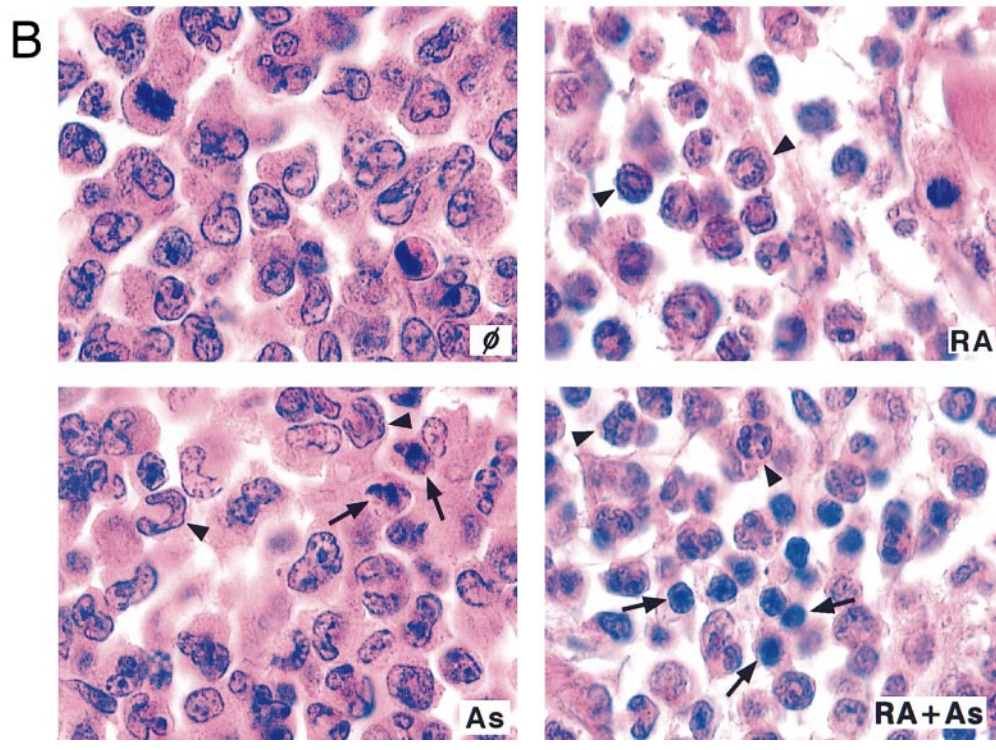
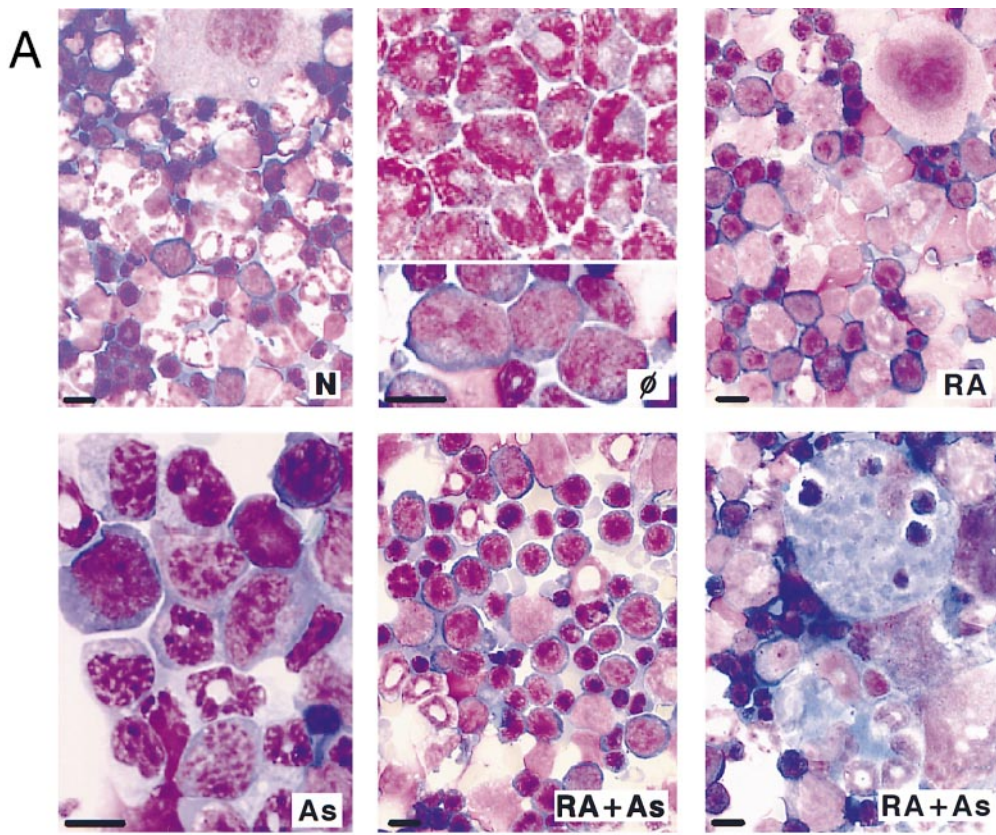
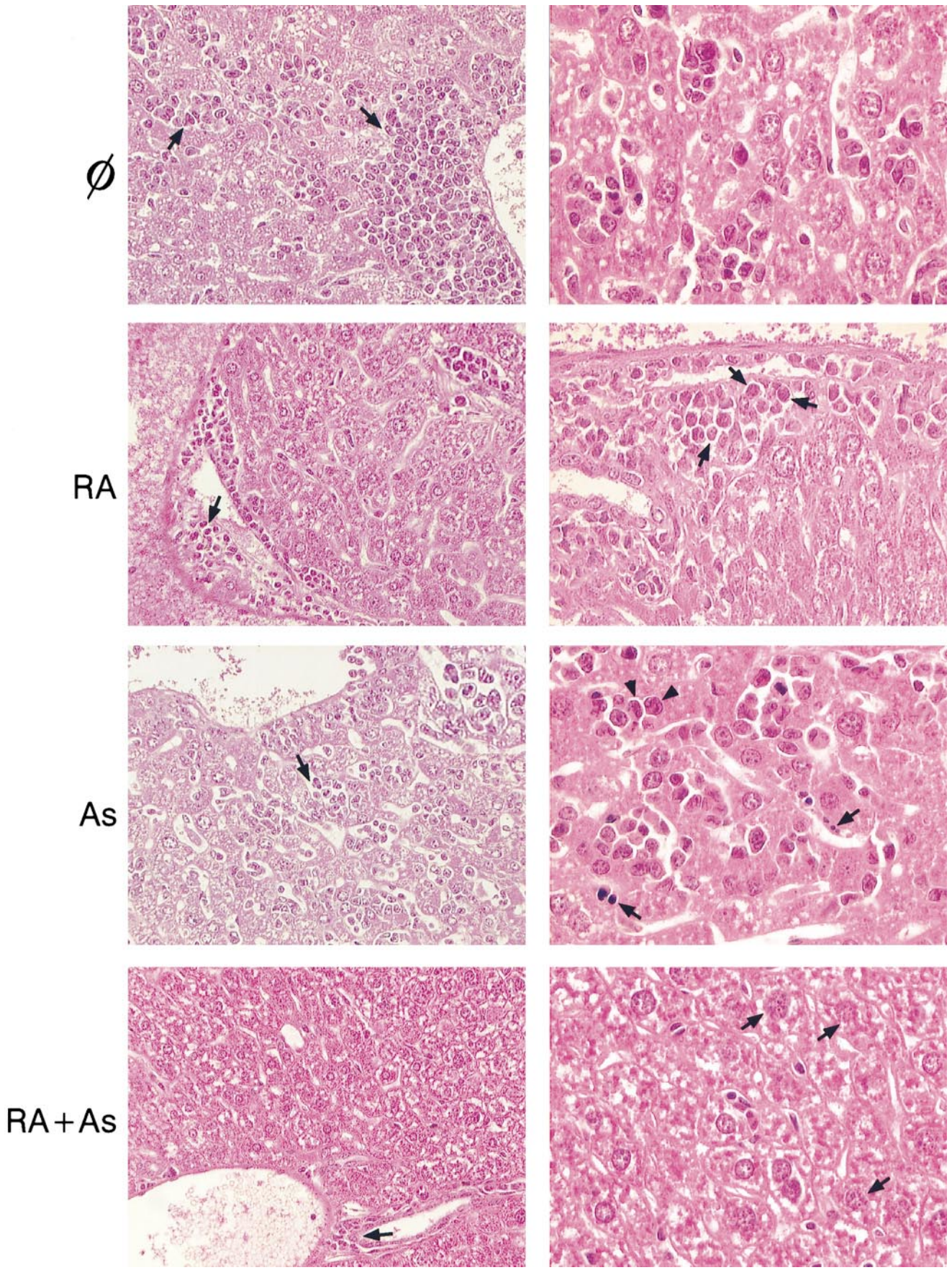


Figure 1. Arsenic and RA induce tumor regression. (A) Cytological analysis by May-Grünwald-Giemsa staining of the bone marrow from leukemic mice left untreated or treated with RA, arsenic, or both for 8 d. N, normal control; Ø, leukemic untreated control; RA, RA-treated; As, arsenic-treated; RA+As, dual therapy. Scale bars, 10 μ m. The bone marrow of the untreated animals is monomorphic. Note that in RA-treated animals, restoration of normal bone marrow is accompanied by a quantitative loss in granulocytes. Arsenic-treated cells exhibit an altered aspect of chromatin. Activated histiocytes with images of phagocytosis (bottom right) are abundant in dual-treated cells. (B) Histopathological analysis of bone marrow of leukemic mice left untreated or treated with arsenic, RA, or both for 4 d. RA induces granulocytic differentiation (arrowheads), arsenic induces apoptotic images (arrows) and differentiation (arrowheads), and their association induces the reappearance of normal marrow elements such as erythroblasts (arrows).

ulocytes compared with nonleukemic bone marrow (Fig. 1 A). In the liver of animals treated with RA for 4 d, small remaining tumor masses consisting of maturing myeloid cells were found around vessels of the portal tracts or cen-

trilobular veins (see arrows in Fig. 2 and Fig. 5 A). Leukemic infiltration of the parenchyme was dramatically reduced at day 8 (not shown). The spleen contained a large number of granulocytes at both 4 and 8 d, but the leukemic infil-



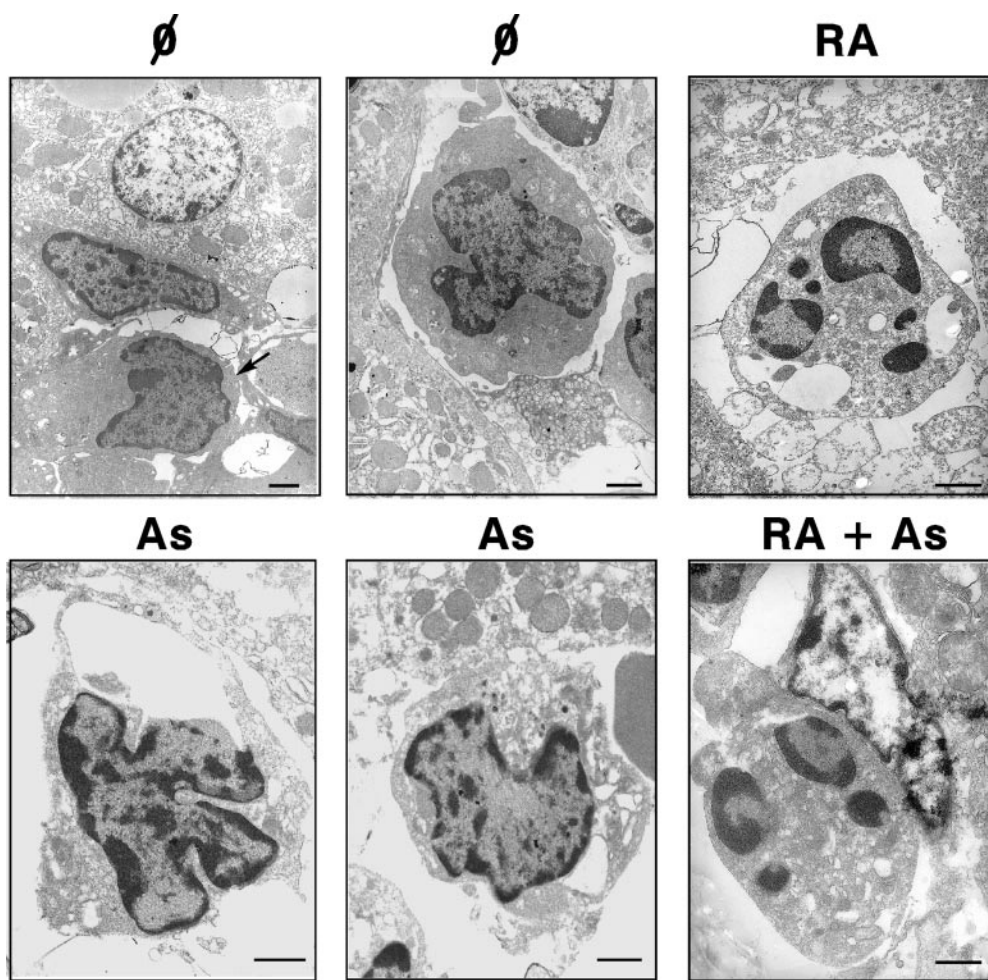


Figure 3. Electron microscopic analysis of liver leukemic infiltrates during various therapies. Ø, leukemic untreated control blasts at low (left) and high (right) magnification; RA, RA-treated cells showing a differentiating myeloid cell; As, arsenic-treated cells showing apoptotic cells with moderately condensed chromatin but no nuclear fragmentation. Note the cytoplasmic lysis. RA+As, dual therapy showing a rare apoptotic granulocyte being phagocytized by a Kupffer cell. Scale bars, 1 μ m.

trate rapidly diminished (not shown). These observations confirm previous analyses of these animals (10).

4 d after arsenic treatment, some cells with a condensed nucleus have apoptotic-like features (arrows, Fig. 1 B), while partly differentiated cells with indented nuclei were also observed (arrowheads, Fig. 1 B). At 8 d, the bone marrow remained quite monomorphic, consisting of myeloid cells with an altered chromatin clearly distinct from that of untreated blasts (Fig. 1 A). In the liver of untreated animals, leukemic blasts infiltrate the parenchyme as very large perivascular masses associated with smaller aggregates of leukemic cells that obstructed sinusoids (Fig. 2, and see Fig. 5 A). In the leukemic blasts from the small intrasinusoid aggregates, arsenic induced morphological changes such as the appearance of indented nuclei and apoptosis-like nuclear condensation (arrowheads and arrows, Fig. 2). As a result of arsenic therapy, only large perivascular masses consisting of differentiated/apoptotic cells remain after the first week (not shown). Nevertheless, at both time points, the reduction in tumor mass was less drastic than that observed with RA.

Treatment with both RA and arsenic led to a much faster decrease in the leukemic population. In the marrow, islets of normal erythroblasts were already clearly visible 4 d after treatment, which was not the case with the single agent treatments (arrows, Fig. 1 B). After 8 d, the bone marrow was normal, with abundant erythroblasts and megakaryocytes (Fig. 1 A). Interestingly, we found numerous activated phagocytes with internalized granulocytes, which could account for the relative deficit in granulocytes compared with nonleukemic marrow. 4 d after treatment, the liver presented only very small remaining aggregates of leukemic cells around large vessels (Fig. 2, and see Fig. 5 A). At 8 d, both liver and spleen appeared tumor-free (not shown).

Mechanisms of RA/Arsenic Synergy. Ultrastructural analysis of liver sections was undertaken to analyze the morphology of leukemic cells after 4 d of therapy (Fig. 3). In livers of untreated leukemic animals, blasts (with lobulated nuclei and dense cytoplasm with some granulations) were clearly visible among hepatocytes and endothelial cells. Upon RA treatment, differentiating myeloid cells resem-

Figure 2. Histopathological analysis of livers after 4-d treatments as indicated. (Left) Low magnification. Arrows point to leukemic blasts. (Right) High magnification. Ø, leukemic untreated control. RA: arrows point to differentiating blasts; arsenic (As): arrowheads indicate differentiating blasts, and arrows point to apoptotic nuclei; RA+As: arrows point to altered hepatocytes.

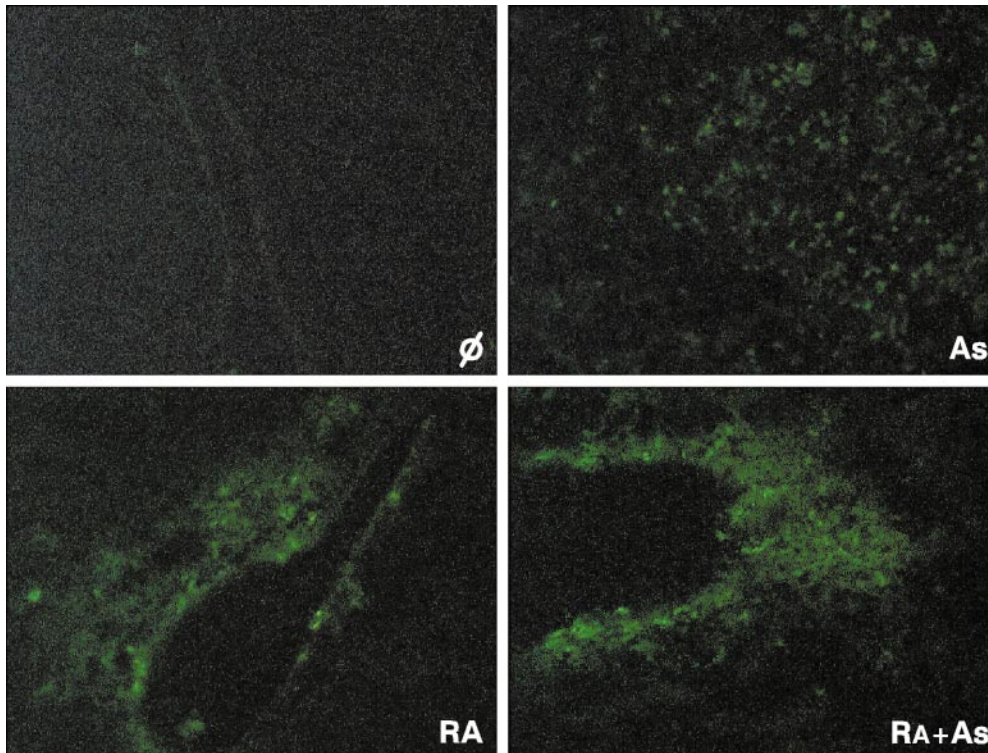


Figure 4. Arsenic and RA induce differentiation. Immunofluorescence analysis of liver sections from mice treated with vehicle (\emptyset), RA, arsenic (As), or both (RA+As) for 4 d. Both therapies induce CD11b expression in vivo, as suggested previously by in vitro studies.

bling granulocytes with fragmented nuclei and dense chromatin were found in the vascular space. Interestingly, arsenic treatment led to the appearance of many cell remnants, often consisting of naked nuclei, or with profound cytoplasmic alterations including large vacuoles and disrupted plasma membrane. However, the chromatin appeared moderately condensed at the nuclear periphery. The nuclear indentations and the presence of cytoplasmic granulations are strongly suggestive for the leukemic origin of these cells. We have recently demonstrated that PML triggers a caspase-independent cell death (7). The aspects of arsenic-treated APL blast unraveled here (Fig. 3) are highly reminiscent of PML-induced death, consistent with the idea that one of the effects of arsenic is to trigger PML-mediated death. Dual-treated specimens harbored very few hematopoietic cells, but on some occasions, images of apoptotic granulocyte phagocytosis were observed (Fig. 3). Altogether, these analyses confirm that RA induces differentiation whereas arsenic triggers a cell death process not associated with major nuclear alterations.

To quantify differentiation and apoptosis, sections were stained with CD11b for assessment of differentiation (22, 29, 30) and a TUNEL assay was used for assessment of apoptosis. Either RA, arsenic, or both treatments sharply induced CD11b expression in the infiltrated liver at day 4 (Fig. 4), as shown previously for APL blasts in patients (29). In the liver, a basal level of TUNEL positivity was noted in the leukemic cells of untreated mice (Fig. 5 A), consistent with high rates of spontaneous apoptosis of tumor cells in vivo. Arsenic sharply enhanced TUNEL positivity, particularly in the small leukemic aggregates in the liver sinusoids

(Fig. 5 A). With RA treatment, intense TUNEL positivity was found in the red pulp of spleen, whereas liver was completely negative, suggesting that RA triggered the migration of differentiated leukemic cells to the spleen where they underwent apoptosis. Double RA/arsenic therapy led to an even more dramatic enhancement of TUNEL positivity in the spleen (Fig. 5 B), suggestive of accelerated differentiation and migration to this site.

RA and Arsenic Cooperate to Induce Complete Remissions. To see whether RA and arsenic also influenced survival, 20 mice were transplanted, allowed to engraft for 12 d, and were then left untreated or were treated with arsenic, RA, or both until the first mouse in the control group died (40 d). Hence, mice were treated for 28 d, and survival was monitored. After arsenic therapy, all animals eventually died within a narrow time range (80 d; Fig. 6 A), as reported above with a shorter implantation time before treatment. In the case of RA therapy, relapses were more scattered but all animals died between 78 and 220 d after transplantation. In striking contrast, all double-treated animals were alive 9 mo after transplantation. The log-rank test demonstrates that differences between the survival of these four groups are highly statistically significant ($P = 0.0001$). Moreover, dual RA and arsenic therapy was significantly better than RA alone ($P = 0.002$). These observations are consistent with the synergistic effects of RA and arsenic on tumor regression.

To know whether the double treatment had actually eradicated the leukemia, surviving animals were killed at day 280 after transplantation. Microscopic examination of the bone marrow and spleen showed no leukemic infiltrate (not shown). The presence of leukemic cells was molecu-

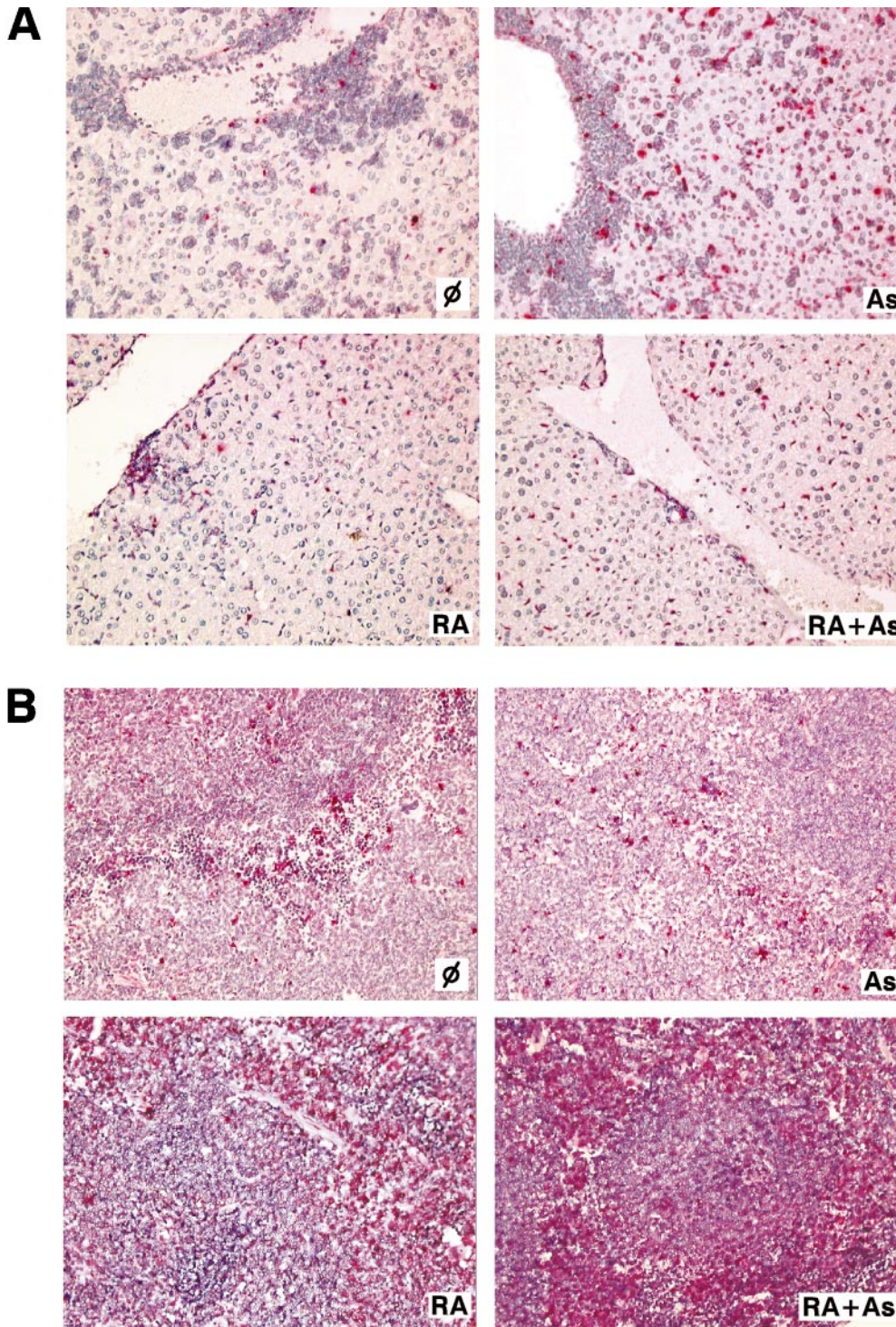


Figure 5. Apoptosis during RA or arsenic therapy. TUNEL analysis of livers (A) and spleens (B) from animals treated as in the legend to Fig. 2 for 4 d. Apoptotic nuclei stain in red; normal cell nuclei stain in blue. Arsenic (As) induces blast apoptosis in the spleen as well as in the liver, whereas RA induces little apoptosis in the liver, but dramatically increases the rate of cell death in the spleen. The association of the two (RA+As) greatly increases apoptosis in the spleen. RA-induced tumor regression is clearly visible (A). Ø, leukemic untreated control.

larly assessed by PCR amplification of the leukemia-specific PML/RAR α fusion gene. In splenic DNA from all four mice tested, no amplification products were found with a nested PCR assay that detects 1 leukemic cell in 1,000–10,000 cells (32; data not shown), whereas the mouse p13 gene was amplified in all four cases. Thus, after dual RA and arsenic therapy, leukemic cells have become undetectable.

Discussion

This report presents evidence that two drugs that specifically target the PML/RAR α fusion protein in APL cooperate in vivo to induce tumor regression and dramatically prolong survival. This model offers the advantage that it closely mimics the APL situation: a population of malignant cells is present in an immunocompetent organism, and only this population is PML/RAR α positive, in contrast to trans-

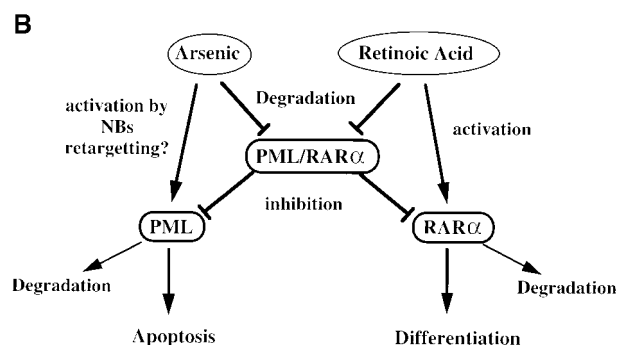
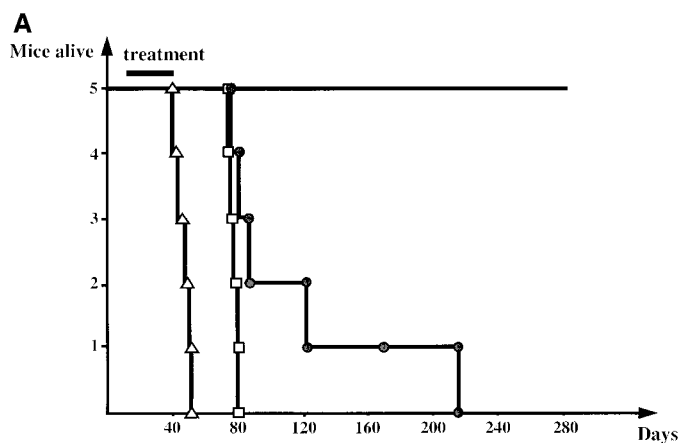


Figure 6. (A) Survival curve of leukemic mice left untreated (Δ) or treated for 28 d with RA (\bullet), arsenic (\square), or both (bold line). The experiment was stopped at month 9. Although single treatments only prolong survival, combining arsenic and RA promotes long-term re-

mission. (B) Model for the synergism between RA and arsenic (adapted from reference 20). Arsenic and RA induce two distinct pathways of PML/RAR α degradation, allowing restoration of PML and RAR α normal functions. Arsenic enhances PML cell death by retargetting the protein onto NBs, and RA activates its receptor to promote myeloid differentiation. PML/RAR α degradation by one agent likely facilitates the action of the other and vice versa.

genic animals where all myeloid cells express the fusion protein. The behavior of the leukemic cells versus the nontransformed hematopoiesis is much better assessed in this setting, and immune response against the leukemia can occur.

Despite previous claims (28), it seems logical that these two drugs which target an oncogene for degradation through distinct pathways cooperate rather than antagonize, confirming our previous findings in vitro (30). A double dominant-negative model was proposed to explain APL pathogenesis, whereby PML/RAR α blocks the functions of the normal RAR α (differentiation) and the normal PML (apoptosis) proteins (20). Apart from inducing PML/RAR α degradation, RA transcriptionally activates RAR, promoting differentiation. In addition, RA induces RAR α degradation (30; our unpublished observations). Similarly, arsenic induces PML/RAR α degradation. Arsenic also targets PML onto NBs, enhancing its proapoptotic properties (7) and subsequently promoting PML degradation (25). Hence, in this double dominant-negative model, PML/RAR α degradation by one agent should favor the action of the other and vice versa (Fig. 6 B). Our results, both in vitro and in vivo showing enhanced differentiation and apoptosis with dual treatments, are consistent with this model. Nevertheless, it is also possible that arsenic modifies the function of RAR α , as it enhances RAR α phosphorylation (25) (which was recently shown to modify its function [33, 34]) and induces RAR α catabolism (30). Together with PML/RAR α degradation, arsenic's effects on RAR α could account for the moderate differentiation induced by this agent. Moreover, the most striking synergy in the double treatments concerns differentiation, suggesting that arsenic enhances RA's effects more than the reverse.

Some toxicity occurred, but under our conditions it was acceptable and never led to deaths. Arsenic alone was hepatotoxic as assessed by moderate edema and steatosis, whereas dual treatment induced some hepatocyte apoptosis suggested by dense rims of nuclear heterochromatin and nu-

clear condensation on electron micrographs (not shown; see also arrows, Fig. 2). Some endothelial toxicity was also noted with dual treatment. However, the absence of major toxicity in a pilot case of dual treatment in a relapse APL patient (Dombret, H., and L. Degos, personal communication) suggests that toxicity is unlikely to limit the association of these two drugs.

In our experimental model, mice relapse quickly after single treatment discontinuation. One obvious possibility is that our treatments were too short. Alternatively, the therapeutic route (subcutaneous for RA, intraperitoneal for arsenic), different from that used in patients (oral for RA, intravenous for arsenic), may not have been optimal. Nevertheless, in human APL, resistance to RA or arsenic as single agents is quite rapid (31, 35, 36). In addition, rate of spontaneous resistance to RA or arsenic of APL cell lines is also high (30, 37). Such high intrinsic resistance of APL cells to these agents could account for the high incidence of relapses with single agent therapy. Here, the apparent eradication of the leukemic clone may reflect the direct differentiating/proapoptotic properties of these two agents. Alternatively, the small number of cells resistant to both RA and arsenic may be eradicated by NK cell activity or by an immune response against the graft. In that sense, the necrotic-like death of arsenic-treated APL cells (Fig. 3) could induce an antileukemia immune response, as proposed in another setting (38).

To our knowledge, these studies represent the first example of clinical trials in a mouse model derived from a transgenic system of a human leukemia. Current protocols use induction therapies based on the simultaneous or sequential use of RA and chemotherapy (39). To date, arsenic is used as a single agent, principally in relapse APL patients (31, 35). The dramatic synergy between these two agents has obvious therapeutic indications: eradication of the leukemic clone in dual-treated animals clearly favors the use of arsenic as a first line drug, suggesting that combined therapies should be assessed in APL patients.

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