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Arsenic Trioxide and Melarsoprol Induce Apoptosis in Plasma Cell Lines and in Plasma Cells from Myeloma Patients[1]

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ABSTRACT

Recent data have renewed the interest for arsenic-containing compounds as anticancer agents. In particular, arsenic trioxide (As_2O_3) has been demonstrated to be an effective drug in the treatment of acute promyelocytic leukemia by inducing programmed cell death in leukemic cells both *in vitro* and *in vivo*. This prompted us to study the *in vitro* effects of As_2O_3 and of another arsenical derivative, the organic compound melarsoprol, on human myeloma cells and on the plasma cell differentiation of normal B cells.

At pharmacological concentrations (10^{-8} to 10^{-6} mol/L), As_2O_3 and melarsoprol caused a dose- and time-dependent inhibition of survival and growth in myeloma cell lines that was, in some, similar to that of acute promyelocytic leukemia cells. Both arsenical compounds induced plasma cell apoptosis, as assessed by 4′,6-diamidino-2-phenylindole staining, detection of phosphatidylserine at the cell surface using annexin V, and by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay. As_2O_3 and melarsoprol also inhibited viability and growth and induced apoptosis in plasma-cell enriched preparations from the bone marrow or blood of myeloma patients. In nonseparated bone marrow samples, both arsenical compounds triggered death in myeloma cells while sparing most myeloid cells, as demonstrated by double staining with annexin V and CD38 or CD15 antibodies. In primary myeloma cells as in cell lines, interleukin 6 did not prevent arsenic-induced cell death or growth inhibition, and no synergistic effect was observed with IFN-α.

In contrast to As_2O_3, melarsoprol only slightly reduced the plasma cell differentiation of normal B cells induced by pokeweed mitogen. Both pokeweed mitogen-induced normal plasma cells and malignant plasma cells showed a normal nuclear distribution of PML protein, which was disrupted by As_2O_3 but not by melarsoprol, suggesting that the two arsenical derivatives acted by different mechanisms. These results point to the use of arsenical derivatives as investigational drugs in the treatment of multiple myeloma.

INTRODUCTION

Recent reports by investigators at the Shanghai and Harbin Institutes of Hematology in China showed that inorganic arsenic trioxide (As_2O_3) is an effective treatment for patients with APL,[3] even for those who relapsed after ATRA-induced remission (1). At the dose used (10 mg/day by i.v. infusion for 28–54 days), no obvious toxicity was observed, including the absence of any significant bone marrow suppression (1). As_2O_3 induces apoptosis of the leukemic cells at a concentration achieved in the plasma of treated patients (0.5–2×10^{-6} mol/L), as demonstrated by studies on ATRA-susceptible or -resistant APL cell lines, on primary APL cell culture, and on patients samples obtained during arsenic treatment (2, 3).

It has been suggested that As_2O_3 might induce apoptosis selectively in APL cells. Indeed, at pharmacological concentrations, it has no effect on the growth and survival of the leukemic myeloid cell lines U937 and HL60 (2). In addition, As_2O_3, like ATRA, leads to the degradation of the PML/RARα fusion protein generated by the specific t(15;17) translocation associated with APL (2). The mechanisms of the degradation of the fusion protein by the two drugs are, however, different (3–5), and whether PML/RARα degradation by As_2O_3 triggers apoptosis by itself or belongs to a spectrum of arsenic-induced cellular changes involved in programmed cell death remains open to question.

Recent preliminary reports suggest that the apoptotic effect of As_2O_3 is not specific for APL cells but can be observed in various cells lines of either myeloid or lymphoid origin (6–8) and in blast cells from patients with non-M3 acute myeloid leukemia (9). Another arsenic-containing compound, the melaminyl-phenyl-arsenoxide melarsoprol, which is used in the treatment of human African trypanosomiasis, has a broad efficacy against leukemic cell lines of both lymphoid and myeloid lineage (10). In particular, the organic arsenic melarsoprol inhibited the growth and promoted apoptosis in cell lines representative of chronic B-cell lymphoproliferative diseases, whereas the inorganic As_2O_3, at similar concentrations (10^{-8} to 10^{-6} mol/L), had no effect on either viability or growth in the three cell lines studied and a minor effect on freshly isolated cells from patients with B chronic lymphocytic leukemia (11).

MM is a B-cell neoplasia due to the proliferation of clonal plasma cells. Remarkable progress has been made recently in the understanding of the pathophysiology of this disease (12). Myeloma cells derive from B cells rescued in germinal centers by an antigen selection process before homing in the BM. Their survival and/or expansion depend on interactions with the BM environment and involve various cytokines, the most important of which is IL 6. Probably because of additional progressive oncogenic events, the disease can progress from an intramedullary to an extramedullary stage, with the occurrence of PCL and/or of visceral involvement. At this later stage, malignant cells may be propagated *in vitro*, giving rise to myeloma cell lines (13). Since the introduction of melphalan, many therapeutic approaches have attempted to improve the survival rate of patients with MM. Although high-dose therapies with hematopoietic stem cell support can prolong duration of remission and survival in young patients, life expectancy remains on the order of a few years, and MM is still an incurable disease (14). This outlines the need for new therapeutic strategies and/or of new active drugs.

We have investigated the effects of both As_2O_3 and melarsoprol on myeloma cell lines and on freshly isolated malignant plasma cells from patients with either the intramedullary or extramedullary stage of MM. We found that the two arsenical compounds, at therapeutical concentrations, markedly inhibit the viability and growth of most MM cells by triggering programmed cell death, with a preferential effect on tumoral cells in patients' BM.

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[3] The abbreviations used are: APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; MM, multiple myeloma; BM, bone marrow; IL, interleukin; PCL, plasma cell leukemia; PWM, pokeweed mitogen; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; Dex, dexamethasone; NB, nuclear body; FACS, fluorescence-activated cell sorter.

MATERIALS AND METHODS

Reagents. A 0.1% As_2O_3 preparation for i.v. administration (kindly provided by G-Q. Chen, Shanghai Institute of Hematology, People's Republic of China) was preserved protected from air at 4°C in aliquots and diluted to a working concentration in RPMI 1640 before use. Melarsoprol (kindly supplied as Arsobal by Rhone Poulenc Rorer, Paris; 36 mg/ml) was stored at room temperature and diluted to a working concentration in propylene glycol. Human recombinant IL-6 and IFN-α were obtained from Diaclone (Besançon, France) and Hoffmann La Roche (Bale, Switzerland), respectively.

Cell Lines. The human myeloma cell lines were purchased from DMS (Braunschweig, Germany; NCI *H929* and LP1) and from American Type Culture Collections (Rockville, MD; OPM2 and U266). NCI, OPM2, and U266 (and all nonplasmacytic cell lines studied) were cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin, 1 mmol/L L-glutamine, and 10% heat-inactivated FCS at 37°C, 5% CO_2 in air. LP1 cells were cultured in similar conditions in Iscove's medium.

Patient Samples. Eleven patients with MM or PCL were studied after informed consent. Mononuclear cells were separated by Ficoll-Hypaque centrifugation from peripheral blood of 4 patients with PCL (including two with a primary PCL), from BM of six patients with MM (including four at diagnosis), and from the pleural effusion of a newly diagnosed myeloma patient. Plasma cell-enriched preparations were obtained by centrifugation of mononuclear cells on a Percoll gradient, as described previously (15). Plasma cell percentages were determined after intracytoplasmic staining using fluorescein-conjugated antibodies to human immunoglobulin heavy and light chains (from Nordic, Tilburg, the Netherlands). Short-term cultures of blood and BM mononuclear cells and of plasma cell-enriched preparations were performed in RPMI 1640 in similar conditions as cell lines.

PWM-induced Differentiation of Normal Lymphocytes. Mononuclear blood cells of three normal donors were cultured in the presence of PWM (from Life Technology, Cergy Pontoise, diluted at 1/100) and of As_2O_3 or melarsoprol (5×10^{-7} or 10^{-6} mol/L). Plasma cells were numerated at day 7 after immunofluorescence staining with fluorescein-conjugated antibodies directed against human immunoglobulin chains. IgM concentrations in supernatants were also measured at day 5, by ELISA, as described previously (16).

Viability, Cell Morphology, and Cell Proliferation. Cells were incubated with or without 10^{-} to 10^{-8} mol/L As_2O_3 or melarsoprol up to 96 h, without any adjustment after culture initiation. Cell number and viability were determined by trypan blue exclusion. Morphological analysis was performed using Wright-Giemsa and 4′,6-diamidino-2-phenylindole staining on cytocentrifuge preparations. For proliferation assays, aliquots of 2×10^4 cells in triplicate were incubated in 96-well plates, and [^{3}H]thymidine (1 μCi) was added for the final 8 h of the culture period. Cells were harvested onto glass filter, and [^{3}H]thymidine incorporation was counted on a liquid scintillation counter.

Flow Cytometry Assay. Detection of phosphatidylserine on the outer leaflet of apoptotic cells was performed using Annexin-V-FLUOS (Boehringer Mannheim, Mannheim, Germany) and PI according to the manufacturer's recommendations. Expression of cell surface CD38, CD138 (BB-4), and CD15 antigens was studied using corresponding FITC or phycoerythrin-conjugated antibodies that were purchased from Diaclone (Besançon, France) and Becton Dickinson (San Jose, Ca.). Flow cytometry was performed with a FACScan apparatus (Becton Dickinson).

TUNEL Assay. Apoptosis was also assessed by dUTP labeling of DNA nicks with terminal deoxynucleotidyl transferase. The TUNEL assay was performed by use of the *In Situ* Cell Death detection kit (Boehringer Mannheim). DNA strand breaks were identified on cytospin slides by labeling free 3′-OH termini with FITC-conjugated dUTP. Incorporated fluorescein was detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with alkaline phosphatase. After substrate reaction, stained cells were analyzed under light microscope.

Immunofluorescence Study of PML. Indirect immunofluorescence staining of PML was performed according to a method described previously (17) using rabbit polyclonal antibodies specific to the NH_2-terminal region of PML (kindly provided by H. de Thé, Saint Louis Hospital, Paris). In some cases, double intracytoplasmic stainings were performed using rabbit antibodies to PML and pig fluorescein-conjugated anti-rabbit immunoglobulin antibodies (Dako, Copenhagen, Denmark), followed by goat rhodamine-conjugated Fab′$_2$ antibodies directed against human immunoglobulin (Nordic, Tilburg, the Netherlands).

RESULTS

As_2O_3 and Melarsoprol Cause a Dose- and Time-dependent Inhibition of Growth in Human Myeloma Cell Lines. The growth of NCI, U266, OPM2, and LP1 plasma cell lines in medium alone or in the presence of As_2O_3 or melarsoprol (10^{-5} to 10^{-8} mol/L) was assessed by cell count and thymidine incorporation. Treatment of NCI cells with different concentrations of As_2O_3 during 72 h induced a marked dose-dependent decrease in cell number and thymidine incorporation with a 50% growth-inhibitory concentration of 3×10^{-7} mol/L (Fig. 1*A*). Fifty % growth-inhibitory concentration was also on the order of 10^{-7} mol/L for OPM2 cells and was about 10^{-6} mol/L for U266 and LP1 cells.

Fig. 1*B* shows the [^{3}H]thymidine uptake of the four plasma cell lines exposed to As_2O_3 10^{-6} mol/L over a time period of 96 h. The arsenical compound also led to a marked time-dependent inhibition of growth in NCI and OPM2 cells and to a significant but less important inhibition of growth in U266 and LP1 cells. As illustrated in Fig. 1, results were similar using the organic arsenical salt melarsoprol instead of As_2O_3.

In addition to the plasma cell type, other hematopoietic cell types were also studied. The growth of the promyelocytic NB-4 cell line was inhibited, whereas inhibition of growth was marginal in T, myeloid, and epithelial cell lines. In contrast, B-EBV cell lines from normal donors were susceptible to both arsenical compounds. Indeed, at 10^{-6} mol/L and after 36 h, As_2O_3 (or melarsoprol) induced a reduction of thymidine incorporation of about 10–20% in Molt 4, HL60, and HELA cells, as compared with ~70% in EBV-immortalized B cells and with 100% in NB-4 and NCI cells.

As_2O_3 and Melarsoprol Reduce the Viability and Induce Apoptosis in Myeloma Cell Lines. Treatment of plasma cell lines with As_2O_3 or melarsoprol led to a dose- and time-dependent inhibition of cell survival, as determined using trypan blue exclusion (Fig. 2). Cell death was mainly due to apoptosis, as shown by the characteristic morphological changes in nuclei as well as by the TUNEL method (Fig. 3). After 24 h of culture with 10^{-6} mol/L As_2O_3 or melarsoprol, a significant percentage of cells exhibiting DNA strand breaks (between 10 and 70% according to the studied cell line, as compared with 3% or less in control cultures) were detected by *in situ* terminal deoxynucleotidyl transferase assay. DNA fragmentation was confirmed by agarose gel electrophoresis of genomic DNA extracted from NCI cells treated with 10^{-6} mol/L As_2O_3 or melarsoprol, which showed typical DNA ladders corresponding to internucleosomal cleavage (not shown).

FACS analysis of NCI and U266 cells after staining with fluorescein-labeled annexin-V and PI was performed to detect the translocation of phosphatidylserine from the inner part of the plasma membrane to the outer layer, which occurs in the early stages of apoptosis (18). Before staining, cells were cultivated with medium alone, As_2O_3 (10^{-6} mol/L), melarsoprol (10^{-6} mol/L), or Dex (10^{-6} mol/L) over a time period of 48 h. As shown in Fig. 4, treatment of NCI cells with melarsoprol rapidly induced apoptosis, as demonstrated by an increasing percentage of annexin-positive, PI-negative apoptotic cells. During the same time, the number of double-positive necrotic cells increased, and at 18 h, only very few double-negative living cells were detectable. Exposure to As_2O_3 induced similar but delayed changes, whereas apoptosis was moderate in cells cultured with Dex (not shown) and in control culture (Fig. 4). Annexin-positive, PI-negative apoptotic U266 cells were also observed in the presence of melarso-

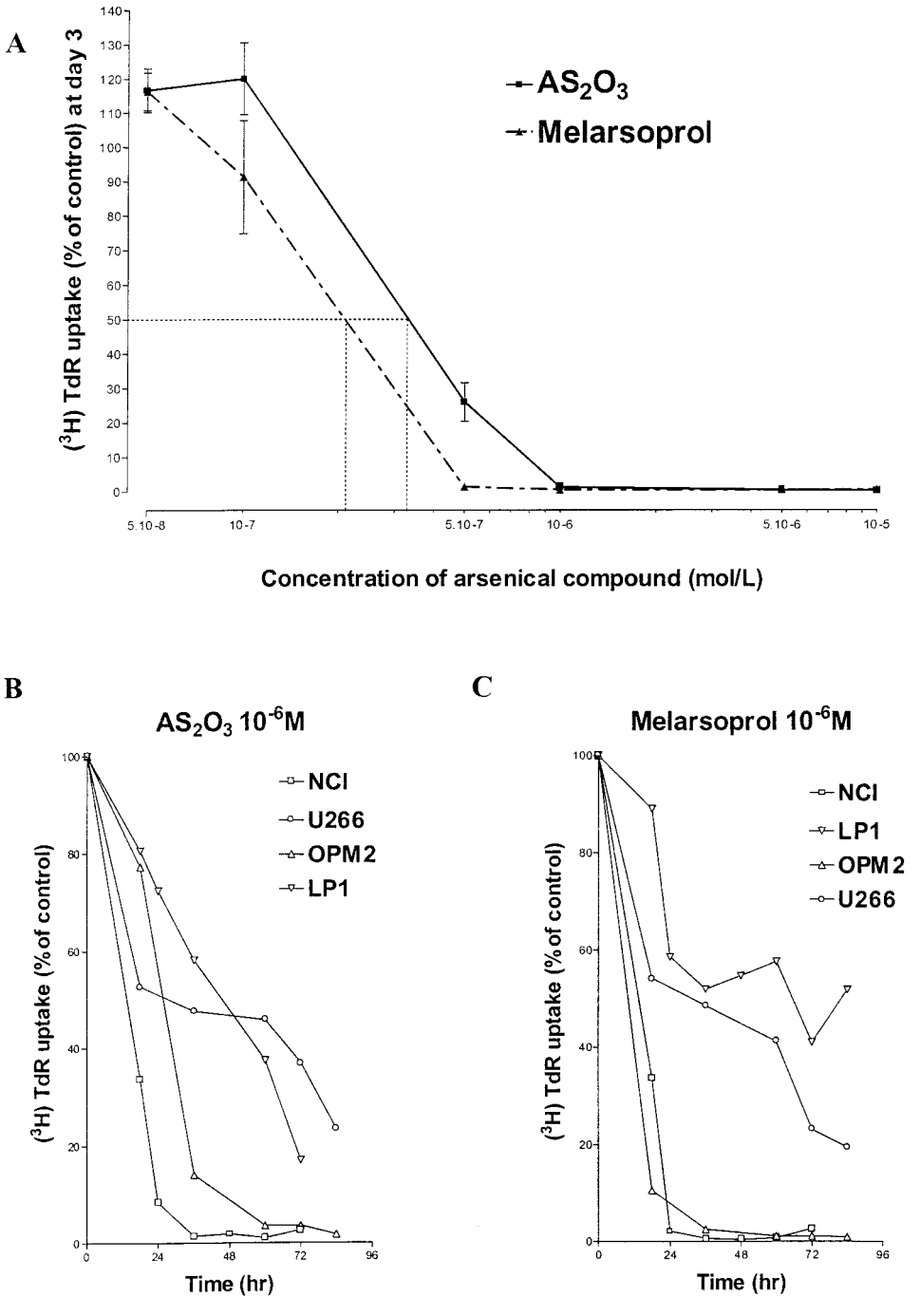

Fig. 1. Dose- and time-dependent effects of As_2O_3 and melarsoprol on the growth ([^{3}H]thymidine uptake) of the different plasma cell lines. *A*, NCI cells cultured during 3 days in the presence of increased concentrations of the two arsenicals. *B* and *C*, NCI, LP1, OPM2, and U266 cells treated with 10^{-6} M As_2O_3 (*B*) or melarsoprol (*C*) for 18–96 h.

prol (18% of residual cells after a 18-h culture) as well as in the presence of As_2O_3 (20.5% at 48 h).

As_2O_3 and Melarsoprol Decrease Viability and Cell Growth and Induce Apoptosis in Fresh Myeloma Cells. As_2O_3 and melarsoprol also inhibited the viability (trypan blue exclusion) and growth ([^{3}H]thymidine uptake) of plasma-cell enriched preparations (containing more than 80% of plasma cells) from BM ($n = 6$), blood ($n = 4$), and pleural effusion ($n = 1$) of 11 myeloma patients. Exposure to 10^{-6} mol/L As_2O_3 and melarsoprol for 48 h induced a mean decline in cell viability to 49 ± 12.3% and 52.5 ± 17.3% (40 to 77.5%, $n = 4$), respectively (Fig. 2). In the three plasma-cell preparations that were cultured in the presence of different concentrations of both arsenical compounds, a dose-dependent inhibition of cell growth was observed, with a 50% inhibitory concentration on the order of 5×10^{-7} mol/L for As_2O_3 and of 10^{-7} mol/L for melarsoprol. A significant percentage of apoptotic cells (up to 50% and 90% at day 2 in the presence of 10^{-6} mol/L As_2O_3 or melarsoprol, respectively, as compared with 5–19% in control cultures) was demonstrated by the TUNEL method in all studied samples ($n = 4$).

Preferential Effect of As_2O_3 and Melarsoprol on Myeloma Cells in Patients' BM. To comparatively assess the effects of As_2O_3 and melarsoprol on myeloid and plasma cells, BM mononuclear cells obtained from patients with newly diagnosed MM were cultured for 5 days in the presence of 10^{-6} mol/L As_2O_3 or melarsoprol. FACS analysis of bone marrow cells after double staining with fluorescein-labeled annexin V and phycoerythrin-labeled CD38 or CD15 antibodies demonstrated that apoptotic cells were mainly myeloma cells. Indeed, in a representative study of a BM sample initially containing

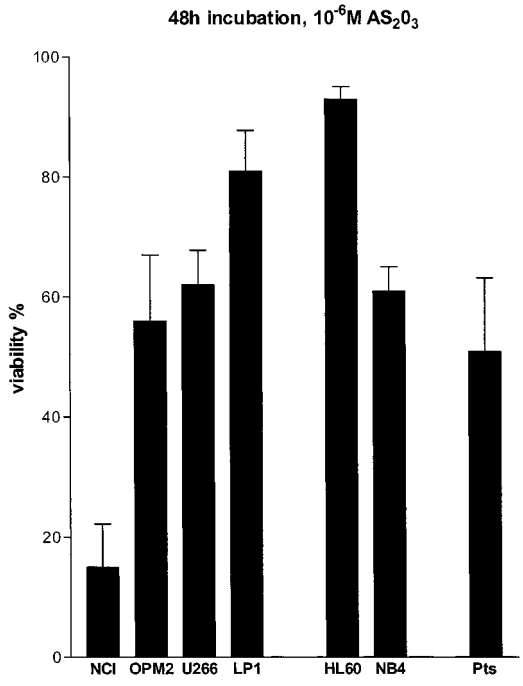

Fig. 2. Viability of plasma cell lines and of plasma cells from myeloma patients in the presence of 10^{-6} mol/L As_2O_3 during 48 h. The percentages of trypan blue-negative cells were 15 ± 7.2, 56 ± 11, 62 ± 5.8, and 81 ± 6.8% in NCI, OPM2, U266, and LP1 cells, respectively. In NB4 and HL60 cell lines used as controls, the percentages of trypan blue-negative cells were 61 ± 4.1 and 93 ± 2.1%, respectively. In myeloma patients (*Pts*, $n = 9$), the cell viability was 51 ± 12.3% (range, 26–64%) as compared with 10.5 ± 14.7% (0–37%) in cultures performed without the drug. *Bars*, SD.

30% plasma cells ($CD38^{high}$ positive), 12% of the latter expressed annexin V after 2 days of culture with 10^{-6} mol/L As_2O_3, whereas <1% of myeloid cells ($CD38^{low}$, CD15 positive) were positive for the apoptosis marker. At day 5, the plasma cell number had decreased much more than the myeloid cell number, and viable cells (PI negative) contained 7.8% of $CD38^{high}$ positive cells, as compared with 22.5% in control culture, performed with medium alone (Fig. 5). Similar results were obtained in the presence of 10^{-6} mol/L melarsoprol (Fig. 5). CD15-positive myelomonocytic cell percentages were similar in arsenic-exposed cultures and in controls, with a decrease in the $CD15^{low}$ positive fraction (Fig. 5).

IL-6 and IFN-α Did Not Influence the Inhibitory Effect of As_2O_3 and Melarsoprol. Because IL-6 has been shown to prevent the apoptosis of myeloma cells exposed to serum starvation or Dex (19), we assayed its effect on the viability and growth of plasma cell lines and primary myeloma cells treated with As_2O_3 or melarsoprol. NCI and U266 cells and plasma cell-enriched preparations from five patients were studied. Recombinant IL-6 alone (100 pg/ml) slightly enhanced the proliferation of all tested cell types. However, whether it was added simultaneously or before the arsenical compound (10^{-6} mol/L to 10^{-7} mol/L), IL-6 did not prevent either cell death or growth inhibition (not shown).

The effect of IFN-α was similarly studied. IFN-α alone (100 or 1000 UI/ml) had a dose-dependent, antiproliferative effect on cell lines as well as on fresh plasma cells. In most cases, the combination of IFN-α with As_2O_3 or melarsoprol had only a minimal effect on the growth inhibition induced by the arsenical compound alone (not shown). Similarly, no synergistic effect was observed by combining the arsenical derivatives and Dex (10^{-6} mol/L; not shown).

Differential Effect of As_2O_3 and Melarsoprol on the Plasma Cell Differentiation of Normal B Cells. Mononuclear cells purified from peripheral blood of normal donors were incubated with PWM in the presence of two different concentrations (10^{-6} mol/L and 5×10^{-7} mol/L) of the organic or of the inorganic arsenical. Fig. 6 shows the percentages of viable plasma cells and the amounts of secreted IgM obtained in a representative experiment. In all cases, melarsoprol, including at 10^{-6} mol/L, only slightly inhibited the plasma cell differentiation induced by the mitogen. In contrast, the number of plasma cells and the amount of secreted IgM were mark-

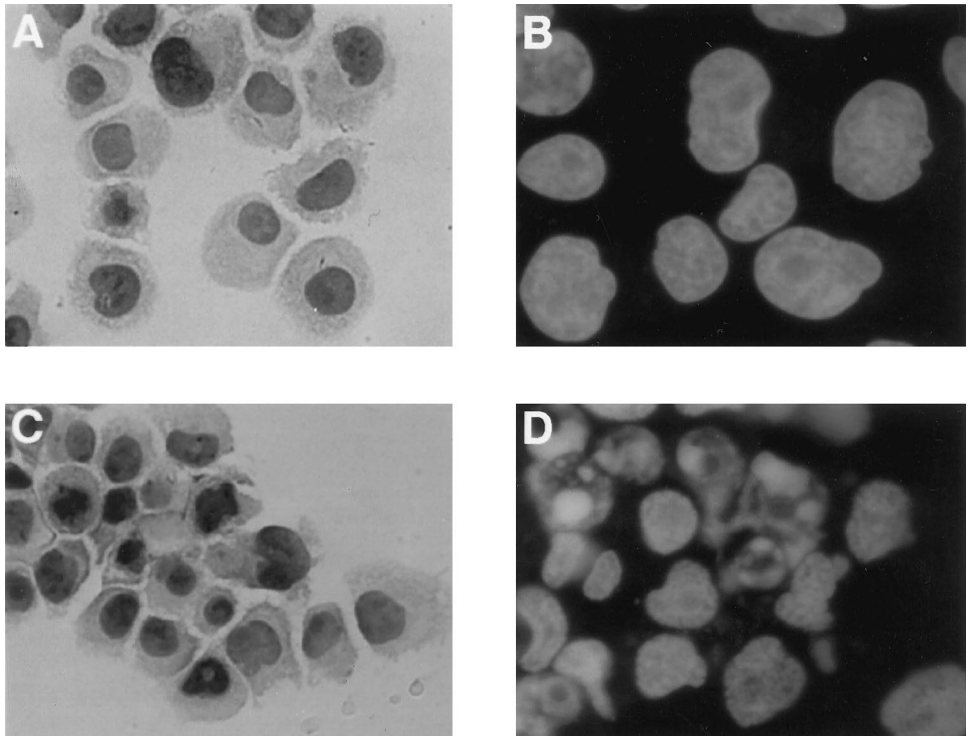

Fig. 3. As_2O_3-induced morphological and molecular changes characteristic of apoptosis in NCI cells. Cytospin slides of NCI cells incubated with (*C* and *D*) or without (*A* and *B*) 10^{-6} M As_2O_3 during 18 h were stained by the 4′-6-diamidino-2-phenylindole nuclear coloration (*B* and *D*) or by an *in situ* TUNEL assay using FITC-conjugated dUTP with detection of incorporated fluorescein by anti-fluorescein antibodies conjugated with alkaline phosphatase (*A* and *C*).

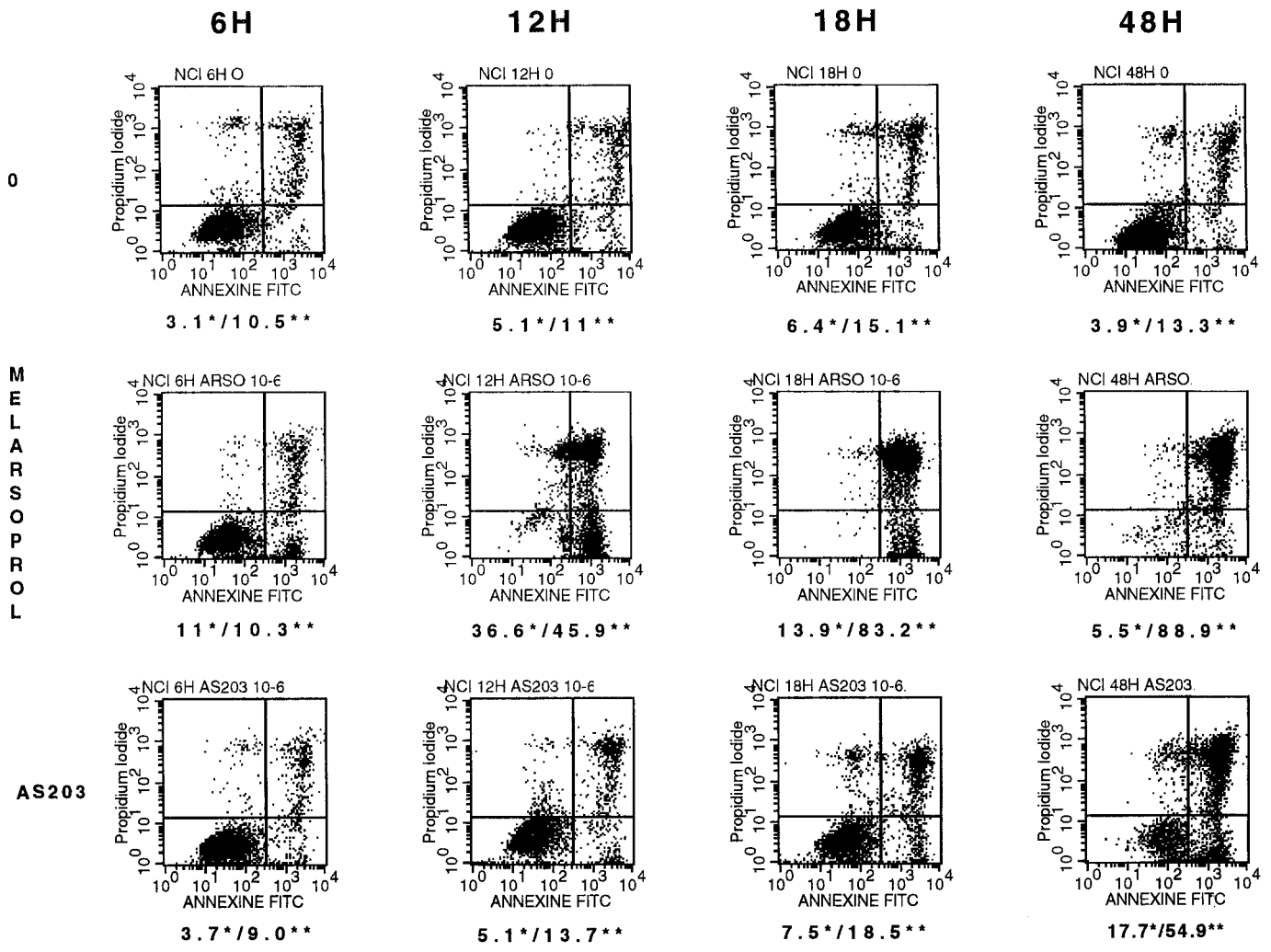

Fig. 4. Two-color FACS analysis of NCI cells after staining with fluorescein-labeled annexin-V and PI. Before staining, cells were cultivated with medium alone (*upper panels*), 10^{-6} mol/L melarsoprol (*middle panels*), or 10^{-6} mol/L As_2O_3 (*lower panels*) for 6, 12, 18, and 48 h. Although cell death was unusually high in the control cultures of this experiment, percentages of annexin-positive, PI-negative apoptotic cells (*) and of double-positive necrotic cells (**) were very significantly higher in arsenic-treated cells, particularly in melarsoprol-treated cells in which apoptosis and necrosis occurred earlier than in As_2O_3-treated cells.

edly reduced in cultures performed with PWM plus As_2O_3, as compared with PWM alone.

As_2O_3 but not Melarsoprol Modify the Nuclear Distribution of PML Protein. The apoptosis induced by As_2O_3 in promyelocytic leukemic cells is associated with a disruption of the macropunctuated pattern of nuclear bodies stained with antibodies to PML (2). To determine whether similar changes occurred in myeloma cells, we stained NCI, U266, OPM2, and LP1 cells with a polyclonal antibody directed against the PML protein. Immunofluorescence staining was performed on cells cultured in medium alone, in the presence of IFN-α (100 or 1000 UI/ml) and/or of As_2O_3 or melarsoprol. In medium alone, the four cell lines showed a typical nuclear speckled staining pattern, which was brighter after a short exposure to IFN-α (Fig. 7*A*). Treatment with As_2O_3 (10^{-6} mol/L) or As_2O_3 plus IFN-α for 24 h led to a marked decrease of the number of PML bodies (Fig. 7*B*). In contrast, melarsoprol did not affect the PML nuclear localization (Fig. 7*C*). Immunofluorescence analysis of the subcellular distribution of PML was also performed in normal peripheral blood mononuclear cells exposed to PWM for 5 days with or without 10^{-6} mol/L As_2O_3 or melarsoprol. To analyze PML in plasma cells, slides were costained with rhodamine-conjugated antihuman immunoglobulin. As in plasma cell lines, the nuclear speckled pattern characteristic of PML disclosed in normal plasma cells was disrupted by As_2O_3 but not by melarsoprol (Fig. 7, *D–F*).

DISCUSSION

In this report, we show that As_2O_3 and melarsoprol reduce the growth and survival of myeloma cells by triggering programmed cell death. The apoptotic effect of the arsenical compounds was observed not only in plasma cell lines but also in malignant plasma cells freshly isolated from patients with MM or PCL, and the arsenic-induced apoptosis was not rescued by IL-6.

These *in vitro* data may prompt study of the clinical efficacy of the two arsenical derivatives in patients with MM, as is the case for As_2O_3 in APL (1). Indeed, As_2O_3 and melarsoprol were active at concentrations ranging from 10^{-6} to 10^{-8} mol/L, which correspond to the *in vivo* pharmacological concentrations of both drugs (1, 20), and some malignant plasma cells were as sensitive to the arsenicals as promyelocytic cells. In contrast, at pharmacological concentrations of As_2O_3 and melarsoprol, reduction in survival and growth of other tested cell types, either lymphoid, myeloid, or epithelial, although significant in some, was less marked than in promyelocytic and plasma cells. The selective effect on myeloma cells of As_2O_3 and melarsoprol was

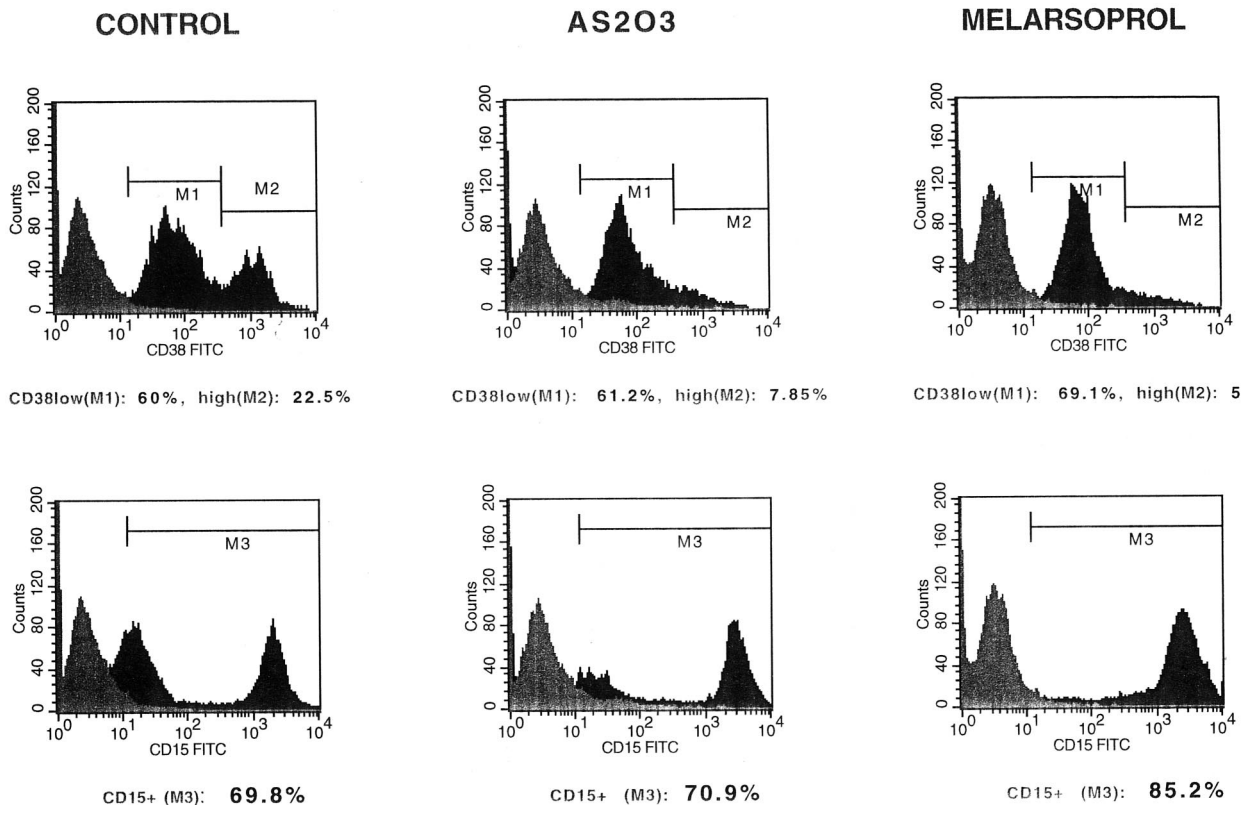

Fig. 5. Expression of antigen CD38 and myeloid marker CD15 on viable (PI-negative) BM mononuclear cells from a newly diagnosed MM patient after a 5 day culture with medium alone (*control*, *left panels*), 10^{-6} mol/L As_2O_3 (*center panels*), or 10^{-6} mol/L melarsoprol (*right panels*). In each panel, the x- and y-axes represent relative mean fluorescence intensity (log scale) and relative number of analyzed viable cells, respectively. Nonspecific staining (*gray* histograms) was defined using isotype-matched control monoclonal antibodies. Both arsenicals induced a striking decrease in $CD38^{high}$-positive (M2) cells, whereas $CD38^{low}$ (M1)-positive cells were spared (*upper panels*). A down-modulation of CD38 antigen expression at the cell surface was ruled out by cytological examinations that confirmed the reduction in plasma cell number. Arsenic exposure did not modify CD15-positive (M3) cell percentages but induced a decrease in $CD15^{low}$-positive cells, especially using melarsoprol (*lower panels*).

illustrated by experiments performed in BM mononuclear cells freshly harvested from patients with MM, which showed a marked decrease in myeloma cell viability with, conversely, only minimal changes in myeloid cells (Fig. 5). These were mainly a decrease in the number of $CD38^{low}$, $CD15^{low}$-positive cells, which include monocytes and myeloid progenitors (21). Of note, semisolid culture assays performed in BM cells from normal donors in the presence of As_2O_3 (up to 10^{-6} mol/L) did not show any inhibition of the number of granulocytic colony-forming cells (data not shown), and no significant myelosuppression was observed in patients treated with As_2O_3 for APL (1).

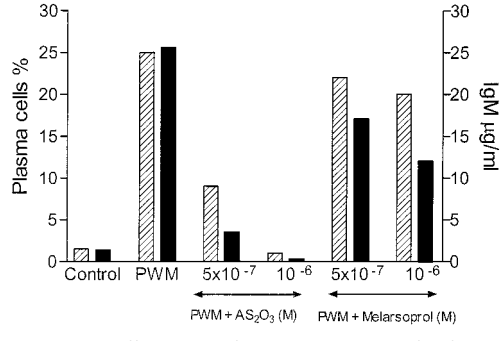

Fig. 6. PWM-induced differentiation of mononuclear cells purified from the peripheral blood of a normal donor in the presence of two different concentrations (10^{-6} mol/L and 5×10^{-7} mol/L) of As_2O_3 or melarsoprol. Viable plasma cells were numerated at day 5 after immunofluorescence staining with anti-immunoglobulin antibodies (▨). IgM concentrations in supernatants were also measured at day 5, by ELISA (■). PWM-induced plasma cell differentiation was inhibited slightly by melarsoprol and more markedly by As_2O_3. Similar results were obtained in blood mononuclear cells from 3 other normal donors.

Similarly, treatment by melarsoprol in patients with African trypanosomiasis does not induce significant cytopenias (22).

König *et al.* (11) recently reported a differential apoptotic effect in EBV or non-EBV cell lines derived from chronic lymphocytic leukemia patients that were sensitive to melarsoprol but resistant to As_2O_3. No similar differential effect was observed in all cell types we studied, including in EBV-transformed B-cell lines derived from normal donors. Results were also similar in plasma cell lines and primary plasma cells, although some (such as LP1 cells) appeared more sensitive to the inorganic arsenical, whereas others (such as NCI cells and most freshly isolated myeloma cells) responded better to the organic one. By contrast, plasma cell differentiation induced in normal B cells by PWM was inhibited markedly in the presence of As_2O_3 and only slightly in the presence of melarsoprol (Fig. 6). This suggests that the two arsenicals may have different mechanisms of action.

A different mechanism of action is also suggested by another striking difference between As_2O_3 and melarsoprol-treated cells, *i.e.*, the nuclear distribution of PML protein. This zinc finger phosphoprotein is expressed in the NBs of normal and malignant plasma cells (Fig. 7), as well as in many normal and neoplastic human tissues (23). In APL cells, the PML/RARα fusion protein generated by the t(15;17) translocation leads to an abnormal micropunctuated distribution of NB proteins that is restored during induction of terminal differentiation by retinoic acid (24). Modifications of PML localization and PML/RARα degradation also occur during As_2O_3-induced apoptosis (4, 5). Similarly, in non-APL cells studied thus far, As_2O_3 recruits the nucleoplasmic form of NB proteins onto NB and induces the degradation of PML (4). As demonstrated here, As_2O_3-induced apoptosis of plasma cells is also associated with changes in PML nuclear local-

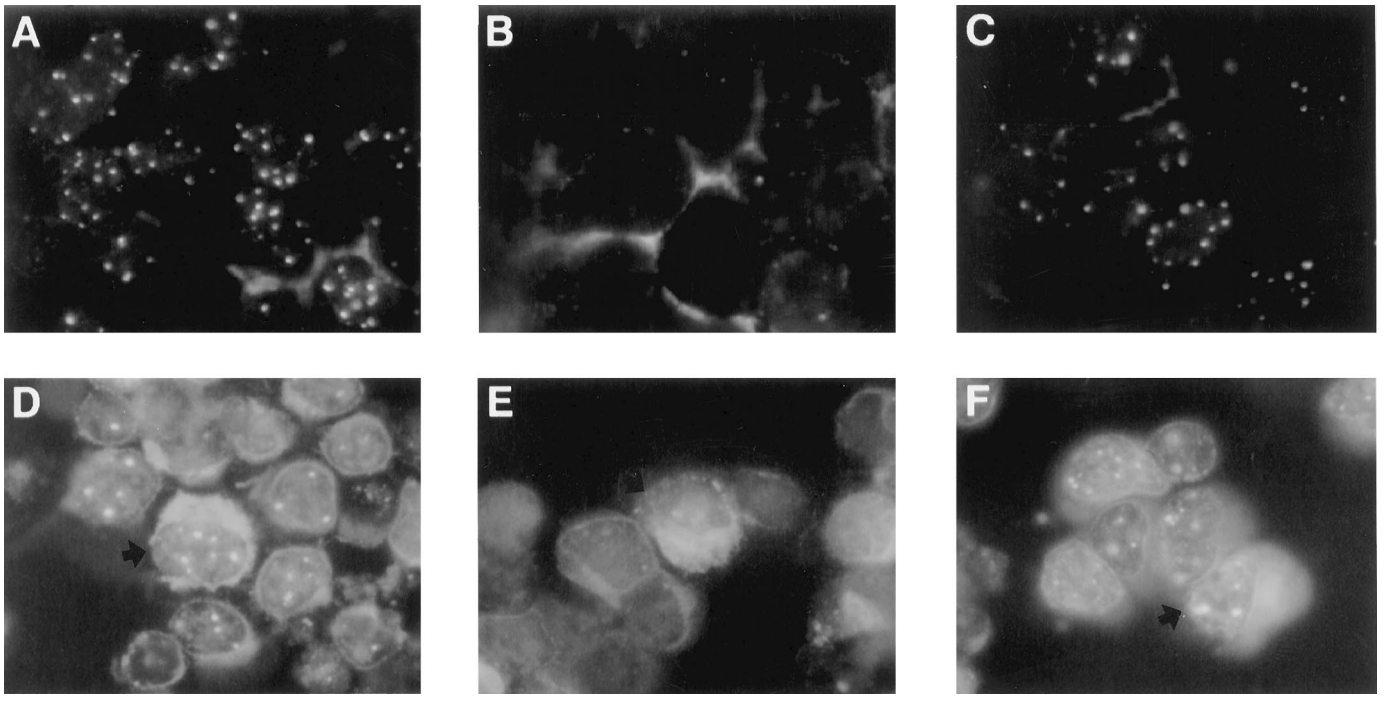

Fig. 7. Indirect intracytoplasmic immunofluorescence staining performed on cytospin slides of NCI cells (*upper panels*) and of peripheral blood mononuclear cells from a normal donor exposed to PWM for 5 days (*lower panels*), using polyclonal antibodies directed against the PML protein and fluorescein-conjugated anti-rabbit immunoglobulin antibodies. PWM-treated cells were costained with rhodamine-conjugated antibodies directed against human immunoglobulin chains. NCI cells were incubated for 3 days with IFN-α (1000 UI/ml; *A*), IFN-α plus 10^{-6} M As_2O_3 (*B*), or IFN-α plus 10^{-6} M melarsoprol (*C*). Normal mononuclear cells were cultured in the presence of PWM without (*D*) or with (*E*) 10^{-6} M As_2O_3 or 10^{-6} M melarsoprol (*F*). In controls (*A* and *D*), most cells showed a nuclear speckled staining pattern typical of PML. Exposure to As_2O_3 led to a marked decrease of the number of PML bodies (*B* and *E*), whereas melarsoprol did not affect the PML nuclear localization either in NCI cells (*C*) or in PWM-exposed normal mononuclear cells (*F*). Among the latter, the differential pattern of PML expression was observed in most cells, including in plasma cells (*arrows*).

ization. By contrast, melarsoprol does not affect the targeting of PML onto nuclear bodies in plasma cell lines as well as in PWM-induced normal plasma cells. Similar results were reported recently in HL60 and U937 myeloid cell lines (25). Accordingly, PML does not appear to be involved in melarsoprol-induced apoptosis, whereas modifications of PML localization might play a role in programmed cell death induction by As_2O_3 (26). It has been shown recently, however, that not only melarsoprol but also As_2O_3 can inhibit growth and induce apoptosis in fibroblasts and BM progenitors in which the *PML* gene has been inactivated by homologous recombination (25).

Arsenic-induced apoptosis of plasma cells was not rescued by IL-6. This cytokine supports the expansion of myeloma cells not only by stimulating cell growth but also by preventing programmed cell death, as demonstrated in several apoptotic conditions such as serum starvation or treatment with dexamethasone or anti-Fas antibodies (19, 27, 28). In addition, blockade of the IL-6 signal, for example using IL-6 receptor (IL-6R) antagonists, can led to apoptosis in myeloma cell lines (29). Similarly, ATRA can act as an apoptotic factor, by down-modulating the IL-6 receptor gp80 and gp130 chain expression and by decreasing IL-6 production by MM cells and BM environment (30, 31). Whether As_2O_3 or melarsoprol could also interfere at any level of the IL-6 pathway remains to be determined. This appears, however, unlikely for melarsoprol because it only slightly inhibits PWM-induced plasma cell differentiation of normal B cells, which requires intact IL-6 circuits (32).

Studies of the effect of arsenical compounds on the bcl-2 apoptosis regulatory family, which includes cell death inhibitors such as bcl-2 and apoptosis inducers such as Bax, provided variable results. Down-regulation of *bcl-2* gene expression was observed either without simultaneous modification of Bax and bcl-X (2) or with down-regulation of other cell death inhibitors (and of some apoptosis inducers) (33). Melarsoprol was also shown to down-regulate bcl-2 in B-cell leukemia lines, both at the mRNA and protein level (11). By contrast, no changes in bcl-2 expression were observed in several cell lines representative of myeloid and lymphoid leukemias in the presence of apoptotic doses of both arsenical compounds (8, 10). Similarly, we found that As_2O_3 and melarsoprol-induced apoptosis of NCI and U266 cells occurred in the absence of any modification in the constitutive expression of the bcl-2 protein (data not shown). These differences may be related to the complexity of the regulation of apoptosis, which depends not only on the expression but also on the phosphorylation and dimerization of the different members of the bcl-2 family (34).

The reactive form of arsenic trioxide is very likely the arsenite (As^{3+}) ion, a sulfhydryl reagent that may interact with free -SH groups on various components of intracellular signaling pathways, as demonstrated for tyrosine phosphatases (35). Arsenical compounds can also produce other reactive agents, such as the arsenate (As^{5+}) ion, which is similar in structure to phosphate and can interfere with phosphorylation-dephosphorylation biochemical reactions. Of note, melarsoprol ($C_{12}H_{15}AsN_6OS_2$), the intracellular metabolism of which is not known, contains only one molecule of arsenic, whereas it is active in myeloma cells at similar molar concentrations as As_2O_3. This additionally argues in favor of different biochemical mechanisms for apoptosis between the two drugs.

Refractoriness of MM cells to presently available therapeutic strategies, even those including high-dose regimens, remains a major problem. Increased intracellular detoxification, mainly by the glutathione system, is a limiting factor for the efficacy of alkylating agents and expression of the M_r 170,000 P-glycoprotein encoded by the multiple drug resistance gene is correlated with resistance to anthracycline and/or vincristine-containing regimens (36, 37). Of note, treatment of cells with As^{3+} diminishes the intracellular level of reduced glutathione (38). In addition, As_2O_3 and melarsoprol may be

active on MDR-expressing cells, as suggested by recent preliminary data concerning doxorubicin-resistant cells, including in the doxorubicin-resistant variant of the RPMI 8266 human myeloma cell line (33).

Although the *in vitro* effects of As_2O_3 and melarsoprol might indicate a clinical efficacy of both drugs in patients with MM, this must be confirmed *in vivo*. Indeed, differential *in vitro* and *in vivo* effects of drugs have been described. For example, the growth of human myeloma cells was inhibited *in vitro* in the presence of ATRA, whereas ATRA was ineffective in the treatment of severe combined immunodeficiency mice transplanted with the same cells (39). We are presently studying As_2O_3 and melarsoprol using similar severe combined immunodeficiency models, and preliminary results appear to confirm *in vivo* the inhibitory effect of both the organic and the inorganic arsenical on MM cell growth. These results additionally point to the use of arsenical derivatives as investigational drugs in MM.

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