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Dimethylsulfoxide-Induced Changes in a Rat Prostate Adenocarcinoma

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A variety of agents can induce mammalian tumor cell lines to acquire characteristics of the normal cell counterpart. Dimethylsulfoxide (DMSO) has been an effective differentiating agent in many tumor cell lines. In the present study a Dunning rat prostate tumor subline, MAT LyLu, available as an in vitro continuous cell culture was treated with 2.25% DMSO (vol/vol).

Treated MAT LyLu cells had a decreased growth rate, saturation density, and clonogenicity, an increased doubling time, and alterations in enzyme activity and tumorigenicity when compared to untreated MAT LyLu cells. The cell viability of treated cells at the saturation density was greater than 90%.

MAT LyLu cells treated with DMSO and then removed from DMSO (posttreated) when compared to untreated cells had similar growth rates, doubling times, clonogenicities, enzyme activities, and tumorigenicities. Posttreated MAT LyLu cells had a different growth pattern than untreated MAT LyLu cells. Posttreated cell viability at saturation density was greater than 90%.

This investigation demonstrated that a rat prostate adenocarcinoma grown in medium containing 2.25% DMSO acquired characteristics consistent with differentiated prostate cells. Posttreated MAT LyLu cells were similar in many characteristics to untreated cells but were not identical. The alterations noted were not cytotoxic and were not completely reversible. The results of this study correlated with the observations of other investigators who have studied mammalian tumor cell lines exposed to DMSO.

Key words: DMSO, differentiation, prostatic cell culture, creatine kinase, lactate dehydrogenase

INTRODUCTION

A variety of agents can induce mammalian tumor cell lines to acquire characteristics of the normal cell counterpart [1]. Polar planar compounds, such as dimethylsulfoxide (DMSO), have been studied extensively and induce differentiation of many tumor cell lines [1–6]. Butyric acid, retinoic acid, hypoxanthine, and actinomycin D have been used as in vitro differentiating agents [1,2,8].

Friend and Freedman [3] studied DMSO-induced differentiation of a murine erythroleukemia. These cells normally are arrested at the proerythroblast level of development and do not differentiate spontaneously. When these cells were grown in media containing 1–2% DMSO (vol/vol) for 4–5 days, approximately 90% of the cells were stimulated to differentiate to the normoblast level and contained hemoglobin levels, delta-amino levulonic acid synthetase levels, and erythrocyte membrane-

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specific antigens consistent with their stage of development. A human promyelocytic leukemia cell line (HL-60), established by Collins and associates [1,2], has been grown in media containing 0.75–1.5% DMSO for 5–7 days. Morphologic differentiation to metamyelocytes and neutrophilic bands and an increased phagocytic capacity occurred in HL-60 cells exposed to DMSO.

DMSO-induced differentiation of cell lines established from solid tumors has also been investigated. Tsao, Kim and associates [6,8] treated two human colon adenocarcinoma cell lines with DMSO. These cells, when grown in medium containing 2.0% DMSO, demonstrated a decreased growth rate, saturation density, clonogenicity, alkaline phosphatase activity, and carcinoembryonic antigen level. The DMSO-induced alterations were considered consistent with the tumor cells acquiring characteristics of the normal cell counterpart. Kimhi and associates [4] studied a murine neuroblastoma supported in media containing 1–2% DMSO and noted a decreased growth rate, saturation density, and tumorigenicity; increased neurite formation and electrical activity; and alterations in acetylcholinesterase and tyrosine hydroxylase activity. These findings were considered characteristic of a more differentiated neural cell.

The research done on hematopoietic cell lines and cell lines established from solid tumors suggests that DMSO might have similar effects on prostate carcinoma cells. In the present study, a rat prostate adenocarcinoma in vitro cell line, when exposed to DMSO, acquired morphologic and biochemical characteristics consistent with a differentiated prostate cell.

MATERIALS AND METHODS

Cell Line

The rat prostate adenocarcinoma used in this study was originated by W.F. Dunning in 1961 [9]. The tumor arose spontaneously in the dorsal lobe of the prostate of an aged, male, Copenhagen rat. The original tumor was a well-differentiated, slow-growing, androgen-sensitive adenocarcinoma of the prostate which had been successfully transplanted, as a subcutaneous tumor, in the flanks of Copenhagen rats [10]. A number of sublines have been isolated from the original tumor [10,11]. The subline of the Dunning rat prostate tumor chosen for investigation was the anaplastic, metastatic (to lymph node and lung), androgen-insensitive cell line known as MAT LyLu [12].

Tumor removed from the flank of a Copenhagen rat was minced with a scalpel into small fragments in a 100-mm tissue culture dish (Corning 25020) containing RPMI 1640 medium (Grand Island Biological Co, Grand Island, NY) supplemented with 20% fetal bovine serum, amphotericin B (2.5 μg/ml), and Garamycin (50 μg/ml). The small fragments were immobilized under a glass coverslip [13], and a monolayer cell culture was established. The cell line was subcultured in the above medium for 30 passages, and, for this investigation, porcine insulin (1 unit/ml) and varying concentrations of DMSO (Fisher Scientific Co, Pittsburgh, PA) ranging from 0 to 4% (vol/vol) were added to the medium. The cells in this new medium were plated in 100-mm tissue culture dishes and subcultured on a weekly basis. The concentration of DMSO that was most effective at causing differentiation was 2.25%. The continuous cell culture used in this investigation was not contaminated with Mycoplasma.

Cell Growth Studies

Cells for growth studies from the tenth and twentieth passages were plated at 5.0×10^4 cells per 60-mm tissue culture dish (Falcon 3002) in new medium and triplicate plates were harvested at 1-day intervals. The number of viable cells was determined by trypan blue dye exclusion on a hemacytometer, and results were plotted on semilogarithmic paper. The slope of exponential growth, doubling time, and saturation density were calculated from this curve. Experiments were performed in duplicate, and the results shown were the mean values.

Clonogenicity

For growth in semisolid agar (Difco Laboratories, Detroit, MI), 1.0×10^3 cells were suspended in 1 ml of 0.3% medium-enriched agar and placed over a 1-ml underlayer of 0.5% medium-enriched agar in a 35-mm tissue culture dish (LUX 5217), as described by Hamburger and Salmon [14]. Cloning efficiency (clonogenicity) in soft agar was determined by counting colonies containing 30 or more cells per colony 1 week after inoculation. Clonogenicity was calculated as the mean of three dishes, and all experiments were performed in triplicate.

Morphology/Histology

Cells grown in tissue culture chamber slides (Lab-Tek 4808) were fixed with formalin and stained with hematoxylin and eosin [15] and for acid phosphatase [16].

Protein/Enzyme Assays

Cells removed from 100-mm tissue culture dishes were centrifuged and washed twice in phosphate-buffered saline (PBS). Cell pellets were suspended in 2 ml of PBS and disrupted by a Polytron homogenizer. Creatine kinase activity was assayed according to recommendations of the German Society for Clinical Chemistry [17] and creatine kinase isoenzymes by the method of Roe and associates [18]. Lactate dehydrogenase activity was determined by the method of Wroblewski and La Due [7] and lactate dehydrogenase isoenzymes by the method of Elevitch and associates [19]. Total protein was calculated by the method of Bradford [20]. All enzyme activities were expressed as international units per gram of protein (IU/g). Mean values were derived from a minimum of six separate determinations. Isotrol (Sigma Co, St. Louis, MO) and lactate dehydrogenase control (Ortho Diagnostics, Raritan, NJ) were used for control isoenzyme distributions.

Cytogenetics

In vitro cells were exposed to Colcemid (0.04 μg/ml; Grand Island Biological Co), for 2 hours at 37°C. Cell preparations were processed as described by Stone and associates [21], and the chromosomes were stained with lacto-aceto-orcein or quinacrine dihydrochloride (Q-banding) for observation and karyotyping. The chromosomes were identified according to the standard karyotype of the Norway rat [22]. The modal chromosome number and standard karyotype were determined from examining 85 to 100 cells.

Tumor Growth in Mice

Athymic nude mice bearing the nu/nu genotype on an outbred Swiss background were bred and maintained in the Division of Laboratory Animal Medicine at the

University of North Carolina at Chapel Hill School of Medicine. Age-matched mice were injected in the right flank subcutaneously with 1.0×10^3 viable cells suspended in 0.2 ml PBS and tumor volume, as calculated by the formula [length $\times$ (width^2)]/2 was determined. Experimental groups contained a minimum of 15 mice, and each experiment was performed in duplicate. The mean tumor volumes of all experiments were plotted on semilogarithmic paper versus time (tumorigenicity). Tumors examined for histology were fixed with formalin, embedded in paraffin, and stained with hematoxylin and eosin [15].

RESULTS

Treated MAT LyLu Cells

Growth studies. In vitro growth curves for MAT LyLu cells grown in media containing 0% DMSO (untreated) and 2.25% DMSO (treated) revealed marked differences in cell growth characteristics (Fig. 1A). The slope of exponential growth for untreated and treated cells (Fig. 1B) was 1.39 and 0.68 ($\times 10^6$ cells/ml/day), respectively, and this difference was significant (F-test, $P < 0.05$). The doubling time for untreated and treated cells was 19.7 and 37.5 hours, respectively, which represented a significant difference (paired t-test, $P < 0.05$; Table I). MAT LyLu cells grown in 0% and 2.25% media had saturation densities of 8.83 and 7.49 ($\times 10^6$ cells/ml), respectively, and these values were not significantly different (paired t-test, $P > 0.05$; Table I). At these saturation densities, there was greater than 90% cell viability, as determined by trypan blue dye exclusion.

Clonogenicity and morphology/histology. The clonogenicity in semisolid agar of untreated and treated cells was 25.0 and 2.0 colonies, respectively, and this difference was significant (paired t-test, $P < 0.05$; Table I). The morphologic and histologic appearance of untreated cells can be observed in Figure 2A. Cells treated with DMSO appeared similar to untreated cells in their morphologic and histologic characteristics. The only morphologic difference was that treated cells had a

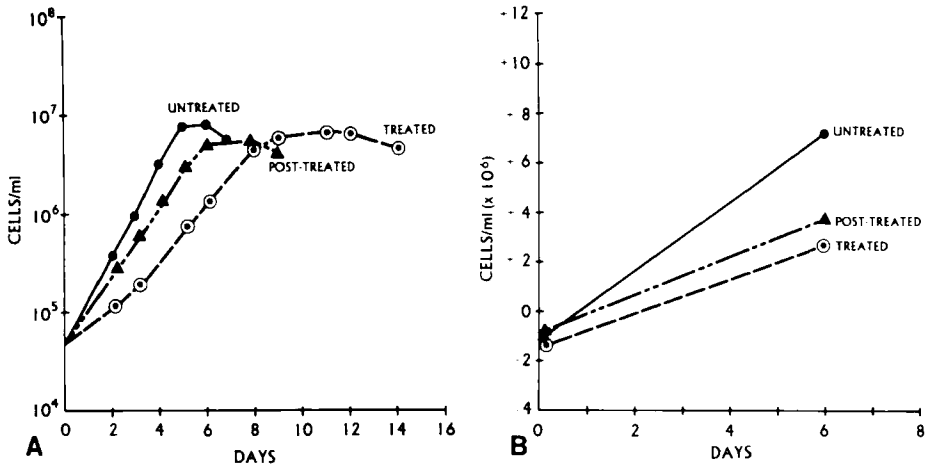

Fig. 1. A) In vitro growth curves of untreated, treated, and posttreated MAT LyLu cells. B) Linear regression analysis of the exponential portion of the in vitro growth curve of untreated, treated, and posttreated MAT LyLu cells.

TABLE I. Effects of DMSO on the In Vitro Characteristics of MAT LyLu Cells

	Untreated	Treated	Posttreated
Doubling time (hours)	19.7	37.5	28.3
Saturation density ($\times 10^6$ cells/ml)	8.83	7.49	5.50
Clonogenicity (colonies)	25.0	2.0	22.0
Creatine kinase activity (IU/g)	57 ± 11	313 ± 51	60 ± 20
Creatine kinase isoenzyme distribution	Minor CK-BB component	Major CK-BB component	Minor CK-BB component
Lactate dehydrogenase activity (IU/g)	5,639 ± 590	5,888 ± 1351	5,912 ± 956
Lactate dehydrogenase isoenzyme distribution	LDH-5	LDH-4 and -5	LDH-5
Modal number of chromosomes (range)	58 (54–65)	58 (52–69)	58 (50–68)

vacuolated, intracellular appearance (Fig. 2B). Untreated and treated MAT LyLu cells had equal amounts of acid phosphatase activity.

Enzymes. Creatine kinase (CK) activity was 57 ± 11 IU/g in the untreated cells and 313 ± 51 IU/g in the treated cells, and this difference was significant (paired t-test, $P < 0.001$; Table I). The CK isoenzyme distribution for MAT LyLu cells treated with DMSO revealed a marked shift to the CK-BB isoenzyme when compared to cells not treated with DMSO (Table I; Fig. 3A). Lactate dehydrogenase (LDH) activity for untreated and treated cells was 5,639 ± 590 IU/g and 5,888 ± 1,351 IU/g, respectively, which was not a significant difference (paired t-test, $P > 0.05$; Table I). Treated cells had detectable levels of the LDH-4 and LDH-5 isoenzymes, but untreated cells had only detectable levels of the LDH-5 isoenzyme (Table I; Fig. 3B).

Cytogenetics. MAT LyLu cells, untreated and treated with DMSO, had similar modal numbers of chromosomes (Table I). The karyotype and six marker chromosomes were not altered by treatment with DMSO (Fig. 4).

Tumor growth in mice. Athymic nude mice were injected with untreated and treated cells, and the differences in tumorigenicity can be noted in Figure 5A. The initiation of tumor growth was delayed by 7 days, the life span was prolonged by 14 days, and the lethal tumor burden was greater in mice injected with treated cells. The slope of exponential tumor growth for mice injected with untreated and treated MAT LyLu cells was 0.73 and 0.67 (cc/day), respectively, and this difference was not significant (F-test, $P > 0.05$; Fig. 5B). The histologic appearance of the solid tumors, as illustrated in Figure 6, revealed no differences between mice injected with untreated or treated cells.

Posttreated MAT LyLu Cells

Growth studies. MAT LyLu cells grown in medium containing 2.25% DMSO for greater than 15 passages were removed from this medium and placed in medium containing 0% DMSO (posttreated). The in vitro growth curve for posttreated cells subcultured in 0% DMSO medium for greater than six passages revealed differences in growth characteristics that were not significant when compared to untreated cells (Fig. 1A). The slope of exponential growth was 1.39 and 0.76 ($\times 10^6$ cells/ml/day) for untreated and posttreated cells, respectively, and these values were not signifi-

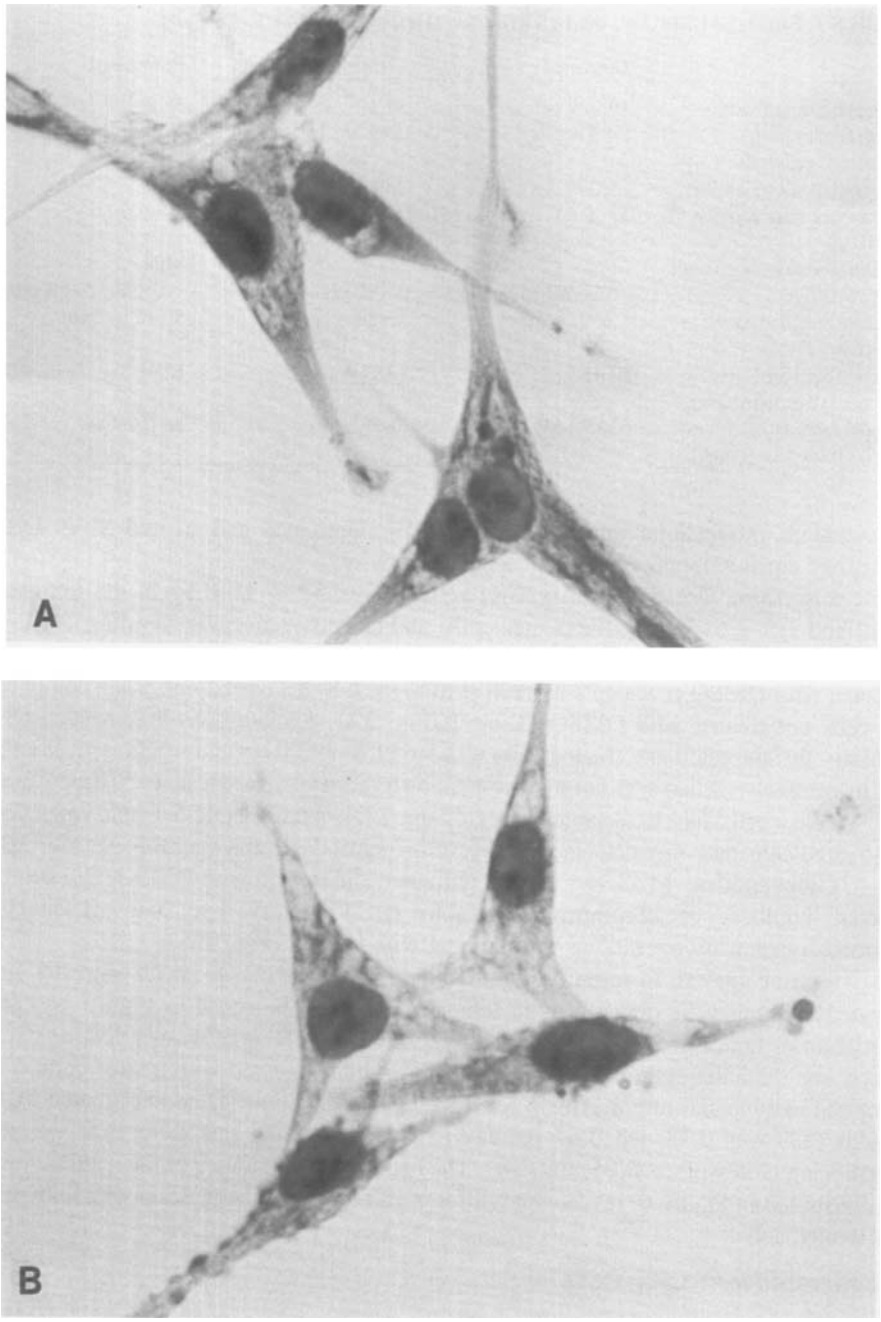

Fig. 2. Photomicrograph of untreated A) and treated B) MAT LyLu cells stained with hematoxylin and eosin. ×1,200.

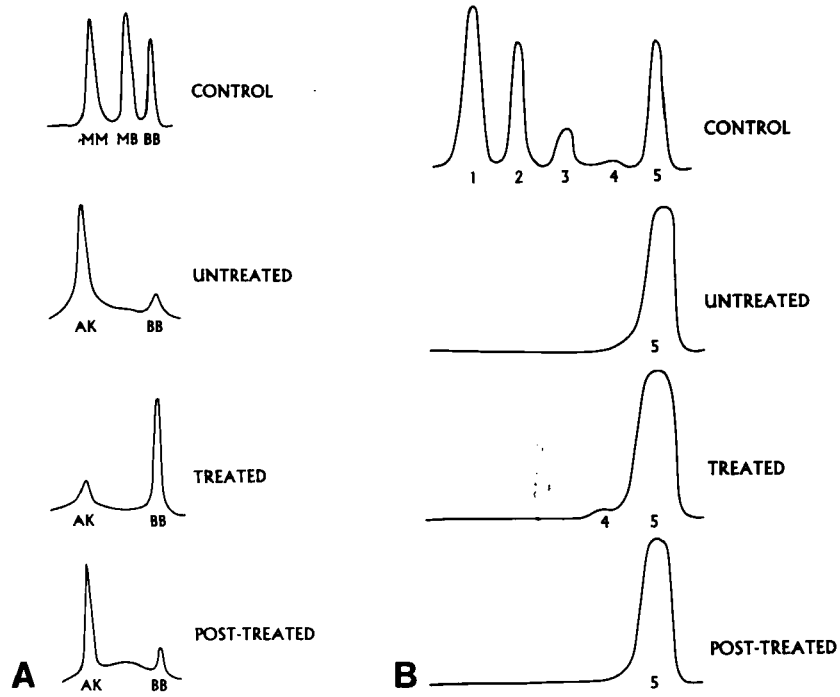

Fig. 3. A) Creatine kinase isoenzyme distribution. B) Lactate dehydrogenase isoenzyme distribution.

cantly different (F-test, $P > 0.05$; Fig. 1B). The doubling time for untreated cells was 19.7 hours and for posttreated cells was 28.3 hours and this difference was not significant (paired t-test, $P > 0.05$; Table I). The saturation densities for untreated and posttreated MAT LyLu cells were 8.83 and 5.50 ($\times 10^6$ cells/ml), respectively, which was not a significant difference (paired t-test, $p > 0.05$; Table I). Cell viability at the saturation density of posttreated MAT LyLu cells was greater than 90%, as determined by trypan blue dye exclusion.

Clonogenicity and morphology/histology. The clonogenicity of untreated and posttreated cells was 25.0 and 22.0, respectively, and this difference was not significant (paired t-test, $P > 0.05$; Table I). Posttreated MAT LyLu cells individually resembled untreated cells (Fig. 2A), but their overall growth pattern was markedly different (Fig. 7). Posttreated cells grew in clumps and were less likely to grow as a monolayer. Untreated and posttreated MAT LyLu cells had similar acid phosphatase activity.

Enzymes. CK and LDH activity for posttreated cells were 60 ± 20 IU/g and $5{,}912 \pm 956$ IU/g, respectively, and these values were not significantly different from those for untreated cells (paired t-test, $P > 0.05$; Table I). The isoenzyme distribution of CK and LDH were similar for untreated and posttreated MAT LyLu cells (Table I; Figs. 3A,B).

Cytogenetics. The modal chromosome number for posttreated cells was 58 (Table I). The karyotypic pattern revealed six marker chromosomes (Fig. 4). These findings were similar to the cytogenetic characteristics of untreated MAT LyLu cells.

Tumor growth in mice. When posttreated MAT LyLu cells were injected into athymic nude mice the initiation of tumor growth and life span were the same as

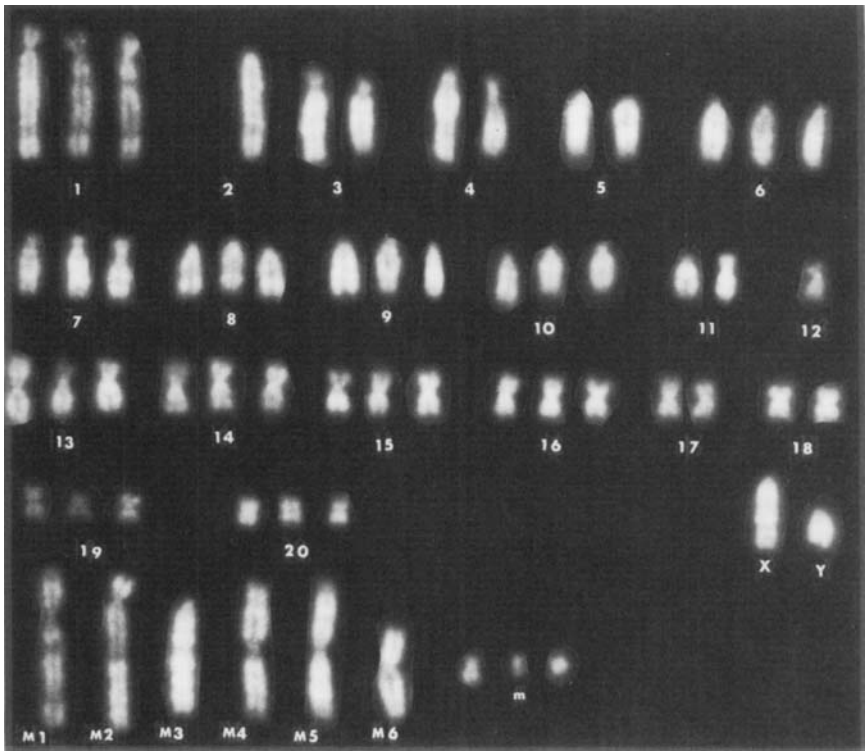

Fig. 4. Q-banded karyotype of untreated MAT LyLu cells. M, marker chromosome; m, minute chromosome. The six marker chromosomes are M1 = t(1q;9q); M2 = t(1p:?;2q); M3 = dir. dup. (2q); M4 = t(6q;7q); M5 = i(5q); and M6 = t(9q;11q).

untreated MAT LyLu cells, but the lethal tumor burden was greater than untreated cells (Fig. 5A). The slope of exponential tumor growth for untreated and posttreated cells injected into athymic nude mice was 0.73 and 0.99 (cc/day), respectively, and these values were not significantly different (F-test, $P > 0.05$; Fig. 5B). The histologic appearance of the solid tumor of posttreated MAT LyLu cells was similar to the solid tumor of untreated MAT LyLu cells (Fig. 6).

DISCUSSION

This study demonstrated that a rat prostate adenocarcinoma grown in medium containing 2.25% DMSO acquired in vitro characteristics of differentiated prostate cells. Treated MAT LyLu cells had in vitro characteristics that correlate with the observations noted by other investigators who have studied the differentiating effects of various compounds [4,6,8,23–25]. The decreased growth rate, saturation density, clonogenicity, and tumorigenicity seen in treated cells have been described as characteristics of a more differentiated state [23,26].

This investigation indicates that the changes noted in MAT LyLu cells treated with 2.25% DMSO were not cytotoxic because cell viability of treated cells was greater than 90%. The increased levels of creatine kinase, shift to the CK-BB isoenzyme, and acquisition of detectable levels of the LDH-4 isoenzyme in treated

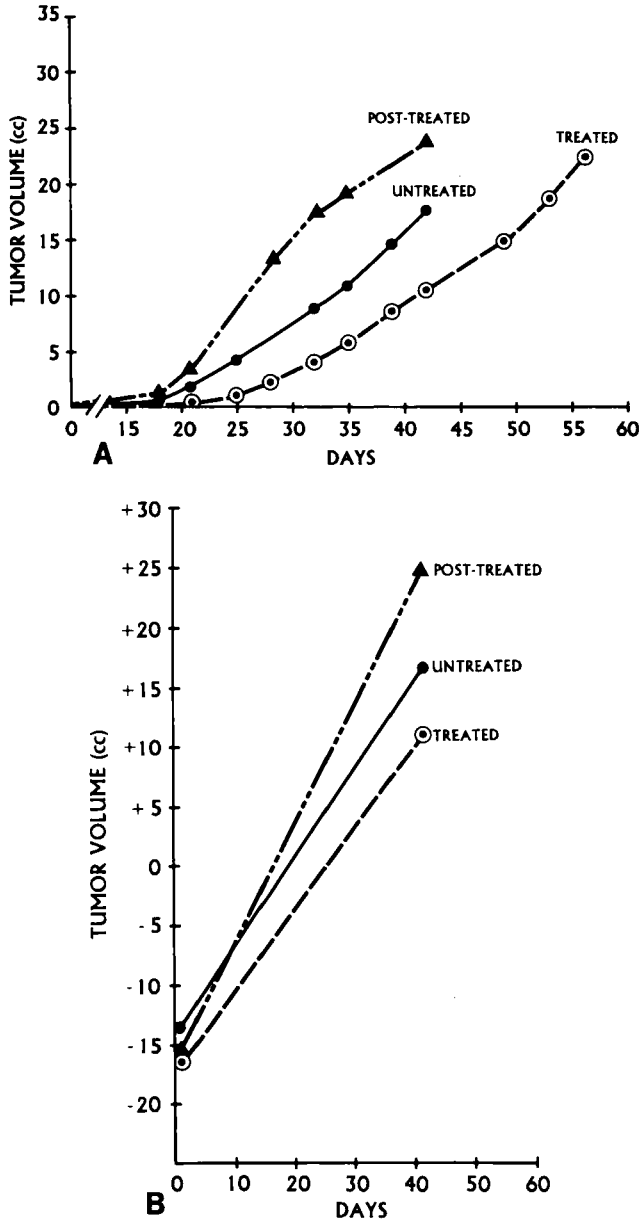

Fig. 5. A) Tumor growth in mice inoculated with untreated, treated, and posttreated MAT LyLu cells. B) Linear regression analysis of exponential tumor growth in mice inoculated with untreated, treated, and posttreated MAT LyLu cells.

MAT LyLu cells confirm that the changes noted were not related to DMSO-induced toxicity. If DMSO was toxic to MAT LyLu cells, a decreased viability and level of enzyme activity would have been observed.

The results of this investigation were not in complete agreement with other studies. A decrease in saturation density has been noted in cells exposed to differentiating agents [6,8,23,24]. Tsoa, Kim, and associates [6,8] noted an approximately

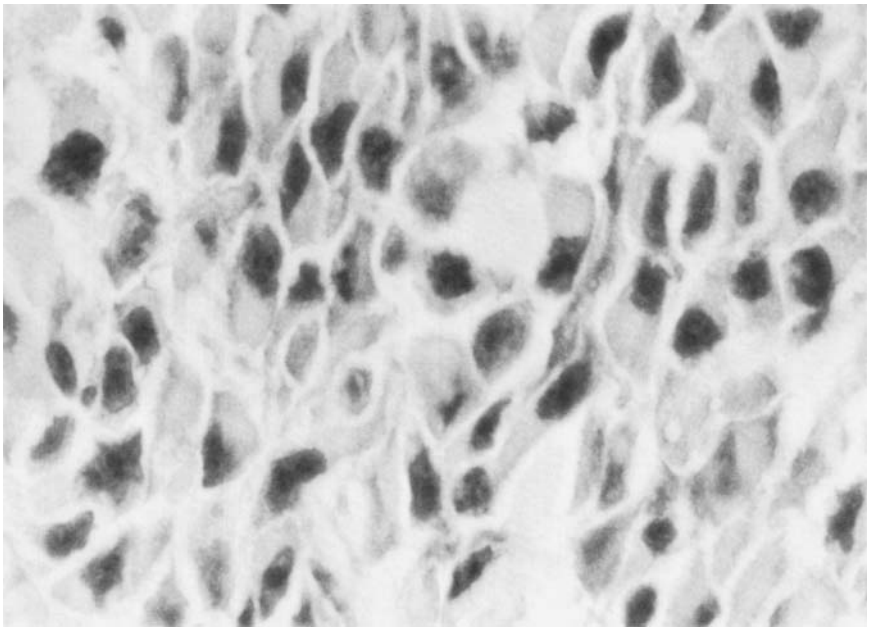

Fig. 6. Photomicrograph of the solid tumor formed in mice injected with untreated MAT LyLu cells stained with hematoxylin and eosin. ×1,280.

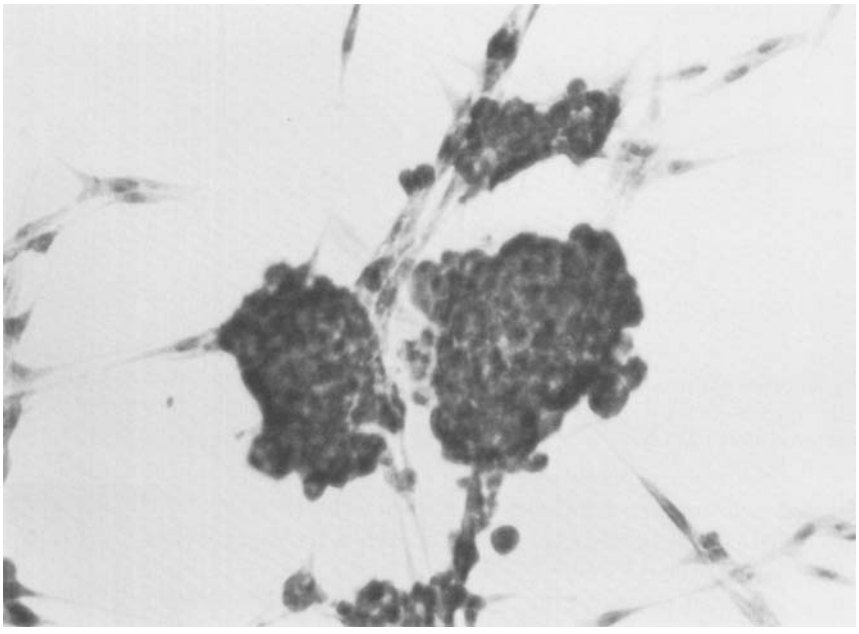

Fig. 7. Photomicrograph of posttreated MAT LyLu cells stained with hematoxylin and eosin. ×320.

sixfold decrease in the saturation density of human rectal adenocarcinoma cells treated with DMSO. Dexter [23] observed an approximately fourfold decrease in the saturation density of murine rhabdomyosarcoma cells treated with N,N-dimethylformamide. In the present study, there was only a 1.2-fold decrease in the saturation density of treated MAT LyLu cells.

The difference in the effect of DMSO on the saturation density of MAT LyLu cells was probably a reflection of the cell line studied and the manner in which cells were treated with DMSO. Many in vitro studies of mammalian tumor cell lines examined the immediate effects of a differentiating agent [4,6,8,23,24]. In the present study, MAT LyLu cells were treated with DMSO for greater than ten passages before the in vitro characteristics of the treated cells were examined. Cells grown in this manner demonstrated the long-term effects of treatment with DMSO.

Many investigators have reported that the effects of DMSO and other differentiating agents are reversible [6,8,24]. Tsoa, Kim, and associates [6,8] observed a resumption of normal growth when DMSO-treated human colon and rectal adenocarcinoma cells were removed from DMSO. Dexter and associates [24] studied human colon carcinoma cells treated with N,N-dimethylformamide and revealed a nearly normal doubling time, clonogenicity, and tumorigenicity when these cells were removed from N,N-dimethylformamide.

In the present study MAT LyLu cells treated with 2.25% DMSO and then removed from DMSO did not completely revert to untreated cells. Posttreated cells had an increased doubling time, an altered morphology and tumorigenicity, and a decreased saturation density. The differences observed might be attributed to a selection by DMSO of a subpopulation of cells from the original cell type [25]. Selection does not appear to be the explanation for the differences noted because if DMSO selected out a particular cell type with differentiated in vitro characteristics, then the posttreated cells would be expected to resemble the treated cells rather than the untreated cells. Selection cannot be ruled out, however. It is possible that selection occurred during the period of exposure to DMSO and testing (ten passages). A second selection could have occurred at the time of posttreatment culture and testing. We feel that this is unlikely because posttreated cells regained many of the characteristics of the untreated cells.

The mechanism of action of DMSO has not been explained and was not the purpose of this study. Friend and Freedman [3] summarized the possible sites of action of DMSO in biological systems as the following: 1) membrane permeability and fluidity, 2) Na^+/K^+-dependent ATPase, 3) Ca^{2+}-transport ATPase, 4) oxygen uptake, 5) enzyme systems, 6) DNA synthesis, 7) DNA and RNA structure, 8) transcriptional function of RNA, and 9) thermostability of chromatin. The exact site(s) of action have not been located to explain the mechanism of action of DMSO as a differentiating agent in mammalian tumor cell lines. The present study indicated no gross changes in chromosomal number or character of treated MAT LyLu cells. There were significant alterations in the enzyme systems of treated MAT LyLu cells. The results observed support many of the possible sites of action of DMSO summarized by Friend and Freedman [3]. An investigation into the effects of DMSO on epidermal growth factor receptor expression and function has been initiated. These studies may help elucidate the mechanism of action of DMSO as a differentiating agent.

CONCLUSIONS

In choosing to work with malignant prostate epithelial cells, we realize that there are no unique differentiation markers. In the studies on Friend erythroleukemia cells, the production of hemoglobin in DMSO-treated cells is unequivocable [3]. One alternative for the prostate system is to find a minimum of two measurable parameters that change in relation to exposure or withdrawal of DMSO. In this study we have demonstrated DMSO-induced changes in creatine kinase activity and in isoenzyme patterns of both creatine kinase and lactate dehydrogenase. Both of these enzymes have been extensively studied in prostate carcinoma. For any malignant cell line in vitro, the classic test for malignant phenotype is ability to grow in soft agar. In the present studies, DMSO exposure rendered the MAT LyLu cells incapable of this malignant phenotypic expression. On withdrawal of the DMSO, the cells were again quite capable of clonal growth in soft agar. Thus, the present investigation revealed that DMSO induced in vitro alterations in MAT LyLu cells that were characteristic of differentiated cells. These alterations were not cytotoxic and were not completely reversible. The results of this investigation support observations noted in other mammalian tumor cell lines grown in median containing DMSO.

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