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T. W. McGarvey
Loyola University of Chicago

B. Persky
Loyola University of Chicago

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THE EFFECTS OF RETINOIC ACID AND BUTYRIC ACID ON IN VITRO MIGRATION BY MURINE B16a CELLS: A QUANTITATIVE SCANNING ELECTRON MICROSCOPIC STUDY

T.W. McGarvey and B. Persky*

Department of Anatomy
Loyola University of Chicago, Stritch School of Medicine
Maywood, IL 60153

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Abstract

Retinoic acid (RA) and butyric acid (BA) were investigated for their effect on in vitro migration of highly metastatic murine B16a melanoma cells. These potential antitumor agents are known to alter the cytoskeleton. Our initial studies determined the 72 h cytostatic/cytotoxic concentration of RA (1×10^{-6} M / > 1×10^{-5} M) and BA (1.5 mM) / > 2.0 mM). Cytostasis by RA and BA was confirmed by autoradiography and radioisotope incorporation. For migration assays, cells were plated on 3 and 5 µm diameter pore polycarbonate membranes. Complete media was added containing RA or BA at time of plating. For BA pretreatment studies, BA was added to cells for 72 h prior to plating cells in fresh BA on the membranes. Top and bottom surfaces of the membranes were examined after 72 h of incubation by scanning electron microscopy. Although RA and BA induced cells on top of the membrane to change morphology as shown by phase, transmission and scanning electron microscopy, only BA enhanced the deformability of cells to allow for passage through the 3 µm diameter pores. Butyric acid enhanced migration through 3 µm diameter pore membranes by 511%. For 5 µm diameter pore membranes, 55.2% of the plated number of untreated early passage cells migrated to the bottom surface as compared to 57.3% for BA-treated cells and 14.9% for RA-treated cells. However, if cellular proliferation over the 72 h period was factored in, BA increased migration by 456% over the controls and pretreatment of cells with BA for 72 h prior to plating increased migration by 893%. Without considering proliferation, RA inhibited migration by 75% over controls. The decrease in migration observed in RA-treated cells was due to an inhibitory effect on cellular migration and a decrease in proliferation.

KEY WORDS: cell migration, butyric acid, retinoic acid, melanoma, murine, B16a cells, in vitro, scanning electron microscopy, cytostasis, cytotoxicity

*Address for correspondence:
Bruce Persky,
Department of Anatomy
Loyola University of Chicago
Maywood, IL 60153
Phone No. (312) 531-4975

Introduction

Progress in the treatment of cancer has been hampered by the inherent ability of tumor cells to metastasize. Metastasis is a complex, multi-step process that is poorly understood. Tumor cells must invade the extracellular matrix (ECM) at multiple stages during the metastatic process. Tumor cell invasion of the ECM has been proposed to be a three step process: (1) attachment to endothelial cells and the ECM, (2) proteolytic digestion of the ECM, and (3) locomotion (Liotta et al., 1983). Although the role of tumor cell locomotion in invasion and metastasis has been studied (Werling et al., 1986; Raz and Ben-Ze'ev, 1987; Maslow, 1987; Verschueren et al., 1988), the specific step of migration needs to be clearly distinguished from the overall process of metastasis (Grimstad, 1987, 1988).

The modified Boyden chamber has been a major instrument to quantitate migration (Varani et al., 1978; Nabeshima et al., 1986; Fliegel et al., 1985; 1986; Wewer et al., 1987). Membrane invasion culture system (MICS) chambers, (Gehlsen et al., 1984), Transwell chambers (Repesh, 1989) and numerous other chambers that are modifications of the Boyden chamber have been used to evaluate migration and invasion. Early studies on quantitating migration in the Boyden chamber (Varani et al., 1978) have now evolved to analyze the effect on migration by various chemotactic agents (Orr et al., 1978; Lam et al., 1981; Thorgeirsson et al., 1982; Varani et al., 1985), haptotaxic agents (McCarthy and Furcht, 1984; McCarthy et al., 1986) and drugs (Spiro and Mundy, 1980; Daughaday et al., 1981). Light microscopy (Thorgeirsson et al., 1982), radiolabelling (Repesh, 1989) and scanning electron microscopy (Albrecht-Buehler, 1986) have been used to evaluate cell locomotion and migration.

Agents that interfere with either microtubules (e.g., colchicine or taxol) or microfilaments (e.g., cytochalasin B) inhibit tumor cell migration (Spiro and Mundy, 1980). Raz and Geiger (1982) speculated that the role of the cytoskeleton was not only active in migration, but also in the tumor cell's ability to deform and make/break intercellular contact. Retinoic acid (RA) and butyric acid (BA) inhibit growth and induce differentiation of tumor cells (Nordenberg et al., 1986; Roberts and Sporn, 1984) as well as affect the cytoskeleton (Lehtonen et al., 1983; Ng et al., 1985; Borenfreund et al., 1980).

In our investigation, we quantitated by scanning electron microscopy (SEM) the migration of

untreated, RA-treated, and BA-treated B16a cells through porous polycarbonate membranes. We calculated migration rates by SEM. The migration rate of B16a cells was defined as the number of cells adhering to the bottom of a polycarbonate membrane divided by the number of cells plated. The number of cells adhering to the bottom of the entire membrane was extrapolated from the number of cells counted within 50 random fields as observed by SEM. This study demonstrated that RA inhibited while BA increased the migration rate of B16a cells. We have also shown by transmission electron microscopy (TEM) that RA and BA induce changes in the cytoskeleton of B16a cells. The role of the cytoskeleton in tumor cell migration is discussed.

Materials and Methods

Cell Culture

Murine B16a (amelanotic) melanoma cells, also referred to as B16-F10a cells, were obtained from the DCT Tumor Repository (NCI Frederick Cancer Research Facility, Frederick, Maryland). Cells were cultured in Eagle's Minimum Essential Medium (MEM) (Gibco, Grand Island, NY) containing Hank's salts and l-glutamine supplemented with sodium pyruvate (Sigma, St. Louis, MO), 10% non-heat inactivated fetal calf serum (FCS) (Gibco), 1% penicillin G-streptomycin sulfate-amphotericin B (Fungizone) (Gibco), MEM non-essential amino acids (Gibco), sodium bicarbonate (Sigma), and Hepes (Sigma). The final pH of the medium was 7.3. Cells were cultured in a humidified incubator (37°C in 5% CO_2) and were refed every three days. Cells were subcultured with trypsin/EDTA (Sigma) during the log phase of growth. All other parameters were standard. The tail vein experimental metastatic assay of Poste et al. (1980) was used to verify the lung-colonizing potential of the cells in C57BL6 mice. The metastatic potential of the cells was confirmed prior to initiating the migration assays and also after completing all assays.

In Vitro Growth Curves of RA, BA, Calf Serum, and Colchicine-Treated Cells

Cells were plated in triplicate for each experiment at a concentration of 150,000 cells per 35 mm dish (50,000 cells per ml). 24 h later, RA (0M, 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M in complete medium containing 0.1% ethanol), BA (0.0mM, 0.1mM, 0.2mM, 0.5mM, 1mM, 1.5mM and 2mM in phosphate buffered saline), 10% calf serum (CS), and colchicine (0M, 10^{-8}M, 10^{-7}M and 10^{-5}M in complete medium containing 0.1% ethanol) were added independently. Retinoic acid and colchicine were obtained from Sigma, BA from J.T. Baker (Phillipsburg, N.J.) and CS from Gibco. Different concentrations of RA were prepared under minimal light conditions according to the method of Lotan and Nicolson (1979). Retinoic acid was dissolved in 100% ethanol to produce a series of stock solutions from 10^{-1} to 10^{-5} M, and stored for up to a week at -60°C. Stock solutions were diluted 1:1000 with complete medium prior to usage. Two sets of controls were run. One control contained complete medium. The second control contained 0.1% ethanol (the final ethanol concentration) in complete medium. The FCS was exposed to ultraviolet light for 1 h in order to destroy vitamin A activity (Ng et al., 1985). The concentration of RA in the control medium was therefore considered to be 0 M. The 10^{-5}M concentration of taxol used in the migration assay was the same concentration used in the motility assay of Keller and Zimmermann (1986). Viability and cell number were determined at 72 h by the trypan blue dye exclusion test and the hemacytometer, respectively.

Incorporation of ^{3}H Thymidine by BA-treated Cells

The incorporation of ^{3}H-thymidine was determined by autoradiography and beta scintillation according to the method of Ryan et al. (1987). For autoradiography, untreated and BA-treated (1.5 mM) were incubated for 72 h on acid-washed glass slides. One μCi/ml of ^{3}H-thymidine (specific activity 6.7 Ci/mM) was added 24 and 54 h after the start of incubation. Slides were washed with cold 0.1 M thymidine in phosphate buffered saline (PBS) and fixed for 2 min at room temperature in freshly prepared 4% paraformaldehyde in PBS (pH=7.4). Slides with unlabelled cells were run as a control. Slides were washed with PBS, dehydrated in ethanol and air dried. They were coated with Kodak NTB2 emulsion, dried overnight in the dark, placed in a light tight box and refrigerated for 2, 4, and 6 days. Slides were developed in D19 developer for 5 min. Each slide was rinsed in 1% acetic acid (30-60 sec) and fixed. Finally, slides were rinsed in running water and stained with Giemsa (Fisher Diagnostics, Orangeburg, NY). Analysis of ^{3}H-thymidine incorporation for autoradiography was done by (1) counting the number of labelled and unlabelled cells, and (2) measuring the percent area of labelling in cells using a PGT digitizer. Background was defined as $\leq$ 25 grains/window on the PGT digitizer at a total magnification of 1550X. The total incorporation of ^{3}H-thymidine using beta scintillation was determined as follows. Control and growth inhibitory concentrations of BA were added in triplicate 24 h after cell plating. After 54 h, 1 or 2 μCi/ml of ^{3}H-thymidine was added for 18 h. Cells were washed with Hank's Balanced Salt Solution (HBSS) without Ca^{++} or Mg^{++} and lifted by trypsin/EDTA. Collected cells were either harvested onto glass filter paper or not filtered. Aqueous scintillation fluor (Packard Instrument Co., Inc., Downers Grove, IL) was added to the filters and to the unfiltered cell suspensions. Counts per minute were determined by liquid scintillation spectrophotometry.

Incorporation of ^{3}H-IdUR by RA-treated Cells

The incorporation of ^{3}H-IdUR was determined by autoradiography and beta scintillation as in the above section with the following exceptions: (1) for both autoradiography and beta scintillation one μCi/ml of ^{3}H-5-Iodo-deoxyuridine (Amersham Inc., Arlington Hts., IL) (specific activity of 5 Ci/mM) was added at 54 h after the start of incubation; (2) the cytostatic concentration of RA was used for both autoradiography and beta scintillation; (3) analysis of incorporation of ^{3}H-IdUR by autoradiography was done only by counting labelled and unlabelled cells; and (4) cellular suspensions were filtered for beta scintillation.

Migration Assay

The methodology in this investigation was a modification of the migration assay of Varani et al. (1978). We utilized either the Transwell chamber system (Costar, Cambridge, MA) or a diffusion chamber (Persky and Grganto, 1987). Transwell chambers have a well within a well design containing a 3 or 5 μm diameter pore polycarbonate membrane

(10 μm in thickness) in the inner well.

Trial 1 In the first investigation, polycarbonate membranes with 3 μm pores were utilized. Cells were plated at least in triplicate in the upper wells of the Transwell chambers (70,000 cells/well; 50,000 cells/well; and 5,000 cells/well). Cells were allowed to attach to the polycarbonate membranes for 24 h prior to treatment with cytostatic concentrations of RA, BA, colchicine, or taxol. Cells were then incubated for three days without refeeding. Drugs were not replenished during the migration assays. Cytostasis was defined as the concentration of drug that inhibited cell growth without measurable cell death. The experiment in which cells were treated with or without BA (50,000 cells/well) was quantitated by SEM (n=4, 500X). The percent migration was determined from the number of cells counted/field times 50 (the number of fields counted) times 15.36 (50 fields at 500X is equal to 1/15.36 of the total surface area). Colchicine, RA, and taxol-treated cells were qualitatively examined.

Trials 2 - 4 In the second investigation, quantification of migration by SEM was done using 5 μm diameter pore polycarbonate membranes (Nuclepore, Pleasanton, CA) in diffusion chambers or 5 μm pore Transwell chambers. Migration was evaluated by counting cells on the bottom surface of the membranes after 18 (Trial 4) and 72 h (Trials 2 and 3) of incubation. The number of migratory cells was determined by counting cells within 50 fields per membrane, each field at 1,700X. This magnification was selected in order to properly delineate cell boundaries. Percent migration was determined by two different methods. The first method was defined as the number of migratory cells counted per field times 50 (the number of fields counted) times 177.59 (50 fields at 1700X is equal to 1/177.56 of the total surface area) divided by 50,000 (the number of cells originally plated). For the 18 h time period, untreated and BA-treated early passage (less than six passages) cells were plated into Transwell chambers (50,000 cells/well). For the 72 h time period, untreated, BA-treated, BA-pretreated (72 h before plating), RA-treated, and calf serum-treated early passage (Trial 2) cells were plated into either the Transwell system or a diffusion chamber (50,000 cells/well). The second methodology for determining percent of migration was calculated only for untreated and BA-treated cells by incorporating a proliferation factor, P_f, for cellular proliferation in 35 mm dishes during the 3 day incubation period. The two P_f values were obtained with a hemacytometer by counting the number of untreated and BA-treated cells after 3 days of incubation. The P_f values were 777,275 and 177,500 for untreated and BA-treated cells, respectively. This method was thus defined as the number of cells per 1,700X field times 50 divided by P_f.

Trial 3 Quantification of migration was done on later passage B16a cells (greater than 6 passages). Untreated, BA-treated, and BA-pretreated (72 h before plating) cells were plated into a Transwell chamber (n=4, 50,000 cells/well).

Scanning Electron Microscopy

All membranes were prepared according to standard techniques except that the membranes were air dried instead of critical point dried. Briefly, membranes were washed in 0.2M cacodylate buffer, fixed in 2% glutaraldehyde, and post fixed in 1% osmium tetroxide. The membranes were dehydrated, air dried, mounted, and sputter coated with gold (27 nm coating; Polaron ES100 Series II sputter coater). Membranes were viewed with a JEOL JSM-840A SEM.

Transmission Electron Microscopy (TEM)

A preliminary ultrastructural study was undertaken to provide evidence that RA and BA modify the cytoskeleton of B16a cells. Transmission electron microscopy was done according to the method of McDonald (1984) and Costar Corporation (personal communication) in order to preserve microfilaments. Briefly, a migration assay was run with untreated, RA-pretreated, and BA-pretreated cells in Transwell chambers. All steps were run with membranes still in the Transwell chambers. Membranes were fixed after 72 h with 2% glutaraldehyde in 50 mM cacodylate buffer (pH=7.4) containing 5 mM $CaCl_2$ for 30 min at 37°C. The membranes were rinsed in buffer (3X) for 5 min at 37°C and incubated in buffer containing 0.8% $K_3Fe(CN)_6$ for 30 min. The membranes were incubated in buffer containing 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$ for 30 min, rinsed in buffer, and then rinsed in distilled water (3X). The membranes were stained en bloc with 2% uranyl acetate (distilled water) for 60 to 120 min, rinsed in distilled water (3X) and then dehydrated in grades of ethanol. Membranes were infiltrated into resin (Epon-Araldite), cut out of the Transwell inner well, and polymerized in blocks at 60°C for several days. Thin sections were cut, stained in 1% uranyl acetate (in 70% methanol) and lead citrate, and viewed with a Hitachi H-600 transmission electron microscope.

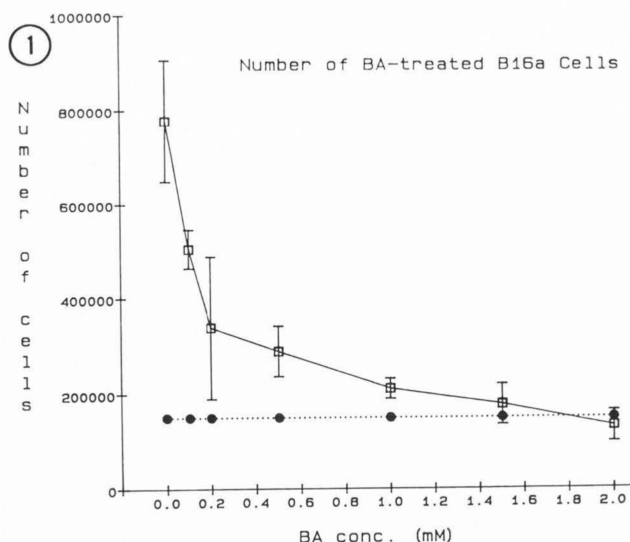
Results

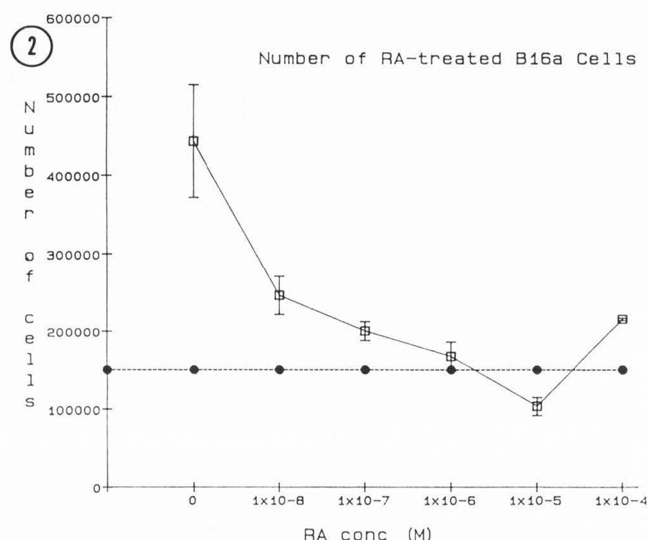
Growth Curves

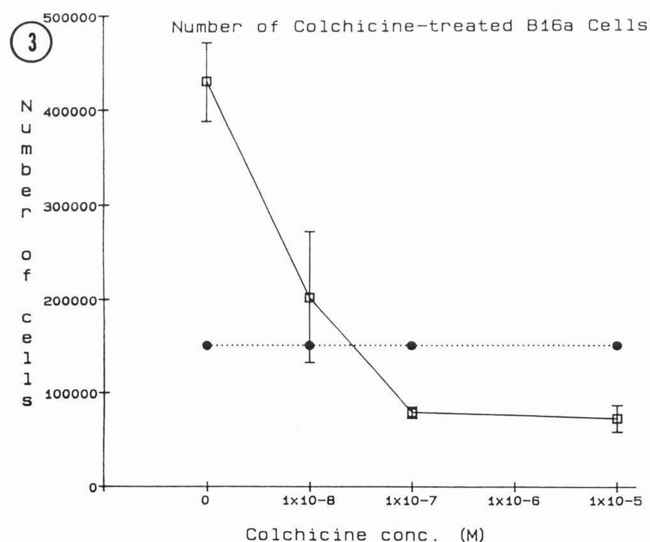
In vitro growth curves of BA (Fig. 1), RA (Fig. 2), and colchicine-treated (Fig. 3) cells were determined by hemacytometer. Cell viability for the six different treatment groups (control medium, control medium + 0.1% ethanol, RA, CS, BA, and colchicine) as determined by the trypan blue exclusion test was always greater than 90%.

Cells were treated for 72 h with different concentrations of BA (Fig. 1). A concentration 2 mM BA was found to be cytotoxic as fewer cells were found after 72 h (130,500 ± 33,600) than were plated (150,000). A concentration of 1.5 mM BA was not cytotoxic yet inhibited proliferation by 77% ± 6% as compared to the controls (0 mM). After 72 h of incubation in 1.5 mM BA, viability was comparable to 72 h controls (0 mM), i.e., 97.3% ± 1.5% and 98.9% ± 0.3%, respectively. The 'cytostatic'concentration of BA was determined to be 1.5 mM and was used for all further cell proliferation and migration experiments. It was also observed that the lowest concentration of BA (0.1mM) inhibited proliferation by 35%.

The cytotoxic/cytostatic concentrations for RA were determined to be 1 X 10^{-5} M/1 X 10^{-6} M, respectively (Fig. 2). The cytostatic concentration inhibited proliferation as compared to the 0.1% ethanol control by 65% and was used for all further cell proliferation and migration experiments. The lowest concentration tested (1 X 10^{-8} M) inhibited proliferation by 50%. The dishes that contained 1 X 10^{-4} M concentration of RA were found to have more viable cells than 1 X 10^{-7} M. Since crystals of RA were found in the 1 X 10^{-4} M concentration medium, we believe that this concentration produced a supersaturated condition such that the RA was not able to







Figures 1-3 (to the left). Dose response curves as measured by hemacytometer for BA, RA, and colchicine-treated B16a cells. Cells were plated (50,000 cells/ml, 3 ml total) in culture dishes. Various concentrations of BA, RA, and colchicine were added after 24 h. Cell number was determined 96 h after plating. Dotted line indicates number of cells plated.

completely dissolve in ethanol. The addition of 0.1% ethanol did not affect proliferation ($p < 0.05$) (Table 1). Proliferation was significantly inhibited ($p < 0.001$) by 59.1% when 10% CS was substituted for 10% fetal calf serum (Table 1).

Figure 3 is the growth curve of colchicine-treated cells. The cytostatic concentration was determined to be 5 X 10^{-7} M.

^{3}H-Thymidine Incorporation in BA-treated Cells

Inhibition of ^{3}H-thymidine incorporation by BA was determined by autoradiography and beta scintillation. In the experiments using autoradiography, two different ^{3}H-thymidine incubation times, 18 h and 48 h, were used. Two separate experiments were done in which the cells were labelled for 18 h, starting 54 h after cell plating. Unlabelled and labelled cells were counted from twelve different fields (average of 125 cells counted/field, 200X) from three different slides (two slides from the first 18 h protocol, one from the second 18 h protocol). Autoradiography revealed that after 18 h 59.1% (standard error of mean, SE = 9.3) of the untreated cells had incorporated the ^{3}H-thymidine, while only 32.9% (SE = 0.6) of the BA-treated cells had incorporated the label. The percent inhibition of ^{3}H-thymidine incorporation was calculated to be 42.0% (SE = 7.1).

Two autoradiography methods (A and B) were used for counting cells that were labelled with ^{3}H-thymidine for 48 h. For both methods, cells were labelled 24 h after plating. For method A, the absolute number of labelled and unlabelled cells was counted. After 48 h of incubation, 96% of the untreated cells were labelled, while 58.5% of the BA-treated cells were labelled (data not shown). The percent inhibition of incorporation of ^{3}H-thymidine was calculated to be 39.1%. For method B, the amount of labelling was calculated on a PGT digitizer from the percent of a set area containing the label (739.8 μm^2, 689X). A total of 53 cells were digitized for each group, i.e., untreated and BA-treated cells. A minimum of 10 random fields were investigated for each group. The area labelled in untreated and BA-treated cells was calculated to be 6.6% (S.D. = 3.2) and 3.2% (S.D. = 3.7), respectively. The percent areas were significantly different ($p < 0.0001$).

Lastly, ^{3}H-thymidine incorporation for untreated and BA-treated cells was measured after an 18 h incubation period by beta scintillation. The average percent inhibition of ^{3}H-thymidine incorporation was 80% (Table 2).

^{3}H-IdUR Incorporation in RA-treated Cells

Inhibition of ^{3}H-IdUR incorporation by RA was determined by autoradiography and beta scintillation. For the autoradiography, unlabelled and labelled cells were counted from 21 (untreated) or 24 (RA-treated) different fields (200X) from three different slides. Autoradiography revealed that 64.5% (SE = 1.58) of the untreated cells were labelled after 18 h incubation with ^{3}H-IdUR, while only 29.8% (SE = 1.47) of the RA-treated cells had incorporated the label. The percent inhibition of ^{3}H-IdUR was calculated to be

TABLE 1 NUMBER OF CELLS AFTER 72 H OF INCUBATION AS DETERMINED BY HEMACYTOMETER

	UNTREATED	0.1% ETHANOL	10% FCS	10% CS
$\bar{X}$	484,343.8	445,500.0	429,750.0	175,916.7
SD	42,932.5	67,752.8	41,511.3	20,779.1
N	4	3	3	3

Table 1. The means of the untreated and 0.1% ethanol groups were not significantly different ($p > 0.05$). The means of the hemacytometer counts for 10% FCS-treated cells and 10% CS-treated cells were significantly different ($p < 0.001$).

TABLE 2 INCORPORATION OF ^{3}H-THYMIDINE CPM AS DETERMINED BY BETA SCINTILLATION

	Unfiltered Control	Unfiltered BA	Filtered Control	Filtered BA
	1 μCi/ul		2 μCi/ul	
$\bar{X}$	630,791.60	202,603.80	1,511,300.0	104,845.2
SD	26,879.85	75,799.01	141,570.6	26,684.5
N	3	3	3	3

Table 2. The ^{3}H-thymidine incorporation by untreated and BA-treated cells after 18 h incubation was measured. Cells were lifted with trypsin/EDTA. Filtered cell suspensions were collected with a cell harvester. The percent inhibition by BA of ^{3}H-thymidine incorporation was 67.9% for unfiltered cell suspensions and 93.1% for filtered cell suspensions. The average percent inhibition was 80%.

TABLE 3 INCORPORATION OF ^{3}H-IdUR CPM AS DETERMINED BY BETA SCINTILLATION

	Filtered Control	Filtered RA
$\bar{X}$	18,898.4	5,232.7
SD	2,371.4	1,032.1
N	3	6

Table 3. The ^{3}H-IdUR incorporation by untreated and RA-treated cells after 18 h incubation was measured. Cell were lifted with trypsin/EDTA and the cell suspension was filtered with a cell harvester. The percent inhibition of ^{3}H-IdUR incorporation by RA was 72.3%.

52.6%. For the beta scintillation, the percent inhibition of ^{3}H-IdUR incorporation was 72.3% (Table 3).

Migration Assay

The initial experiments using 3 μm diameter pore polycarbonate membranes led to further analysis and quantification of migration with 5 μm diameter pore membranes. Light microscopy showed untreated cells grown on plastic to be heterogeneous in morphology (round to flat) as well as diverse in cell size. In contrast, BA-treated cells were a homogeneous population of large, flattened cells.

The morphology of untreated cells (Figs. 4 and 5), RA-treated (Fig. 6), BA-treated (Fig. 7), colchicine-treated (Fig. 8) and taxol-treated (Fig. 9) cells was also evaluated by SEM on both 3 and 5 μm diameter pore polycarbonate membranes. In all cases, cells on the top surface of the polycarbonate membranes had morphology similar to comparable treated cells grown on plastic. Butyric acid (Fig. 7), colchicine (Fig. 8), and taxol (Fig. 9) treatment induced a flattened morphology as compared to untreated cells whether the cells were grown on plastic or on polycarbonate membranes. Colchicine and taxol-treated B16a cells on top of the membrane were not as flattened in shape as BA-treated cells. The majority of colchicine (Fig. 8) and taxol treated cells (Fig. 9) were covered with blebs. Retinoic acid induced a spindle-shaped morphology. The major observation was that many more BA-treated cells migrated through 3 μm pore membranes than untreated, RA, colchicine, or taxol-treated cells. The experiment in which cells were treated with or without BA (50,000 cells/well) was quantitated using SEM. The increase in the percent migration induced by BA over untreated controls was calculated to be 511% (Trial 1, Table 4). Migration through the membrane for untreated (Fig. 4), RA-treated, colchicine (Fig. 8) or taxol (Fig. 9) was < 2% of the original plated number. Cell plating density, which ranged from 5,000 to 75,000 cells per well, did not have an effect on the migration of untreated and BA-treated cells through the 3 μm pore polycarbonate membrane (data not shown).

Later experiments quantitated by SEM the migration of untreated and treated cells through 5 μm pore diameter membranes after 18 (Trial 4, Table 4) and 72 h (Trials 2 and 3, Table 4) of incubation. Both diffusion chambers and Transwell chambers were used. Table 4 illustrates that Transwell chambers were used for Trials 3, 4, and PBA, RA, CS treatments of Trial 2. Diffusion chambers were used for the other parameters of Trial 2.

Trial 2 Using early passage cells (passage less than six), an average of 3.11 control cells/field (SE = 1.22) and 3.23 BA-treated cells/field (SE = 1.13) were observed on the bottom of the membrane

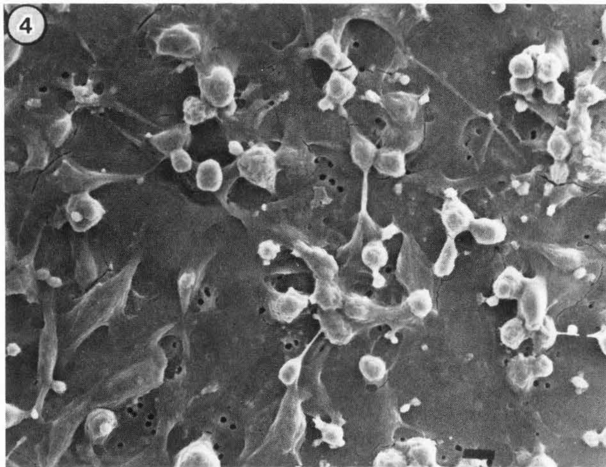
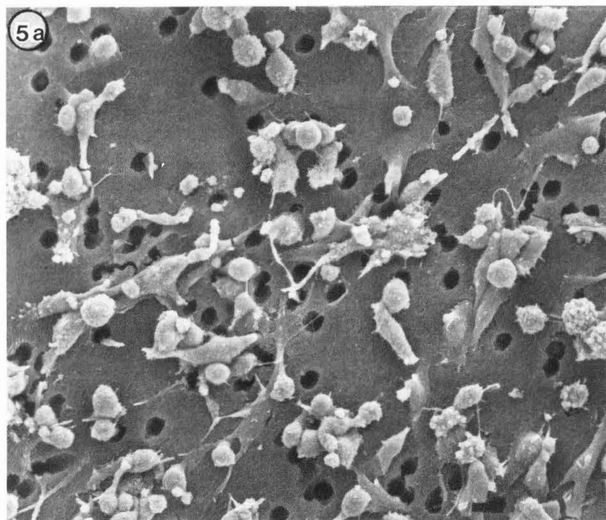


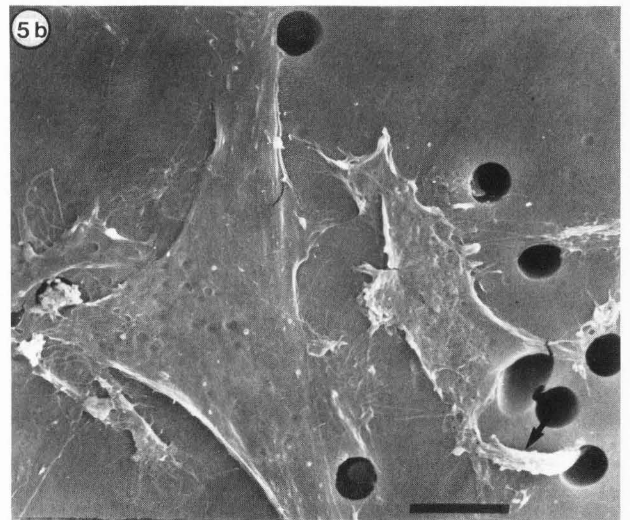
Figure 4 (at left). Scanning electron micrograph of untreated cell on the top surface of a 3 μm pore polycarbonate membrane after 72 h of incubation.

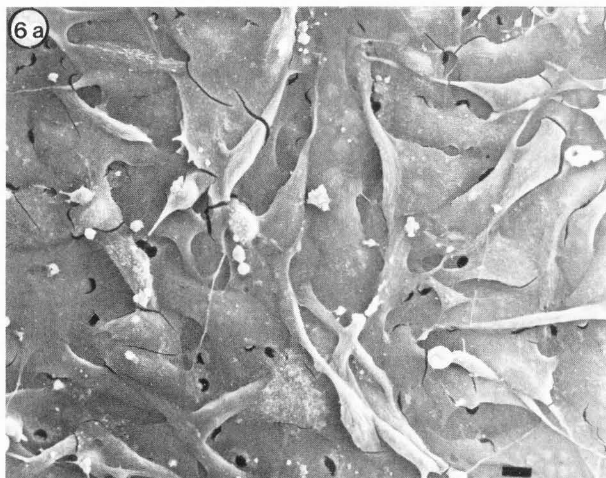
Figures 5-7 (below and facing page top). Scanning electron micrographs of untreated (Fig. 5), RA-treated (Fig. 6), and BA-pretreated (Fig. 7) cells on the top ("a") and bottom ("b") surfaces of 5 μm diameter pore polycarbonate membranes after 72 h of incubation. Note the pseudopod coming out of a pore in Figures 5 and 6 (arrow).

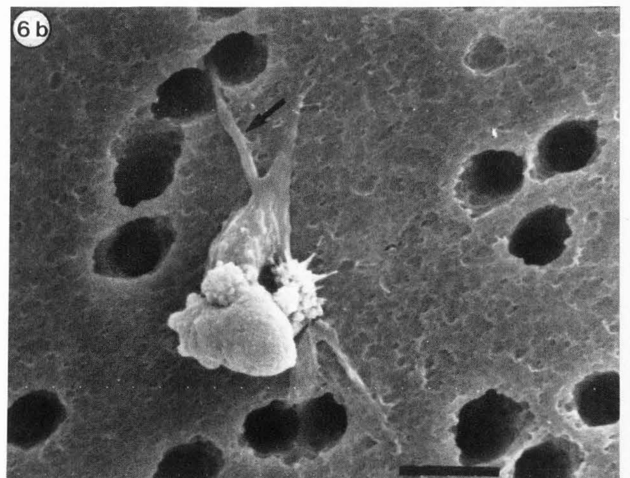
Figures 8-9 (facing page middle). Scanning electron micrographs of colchicine and taxol-treated cells on the top surface of 5 μm diameter pore polycarbonate membranes after 72 h of incubation.

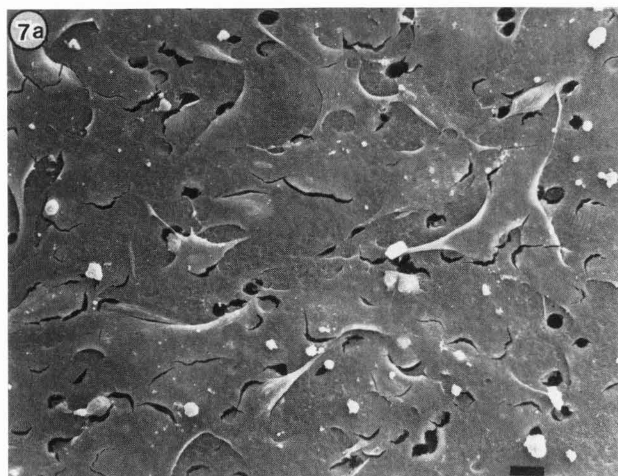
Bars = 10 μm for Figures 4 to 9.

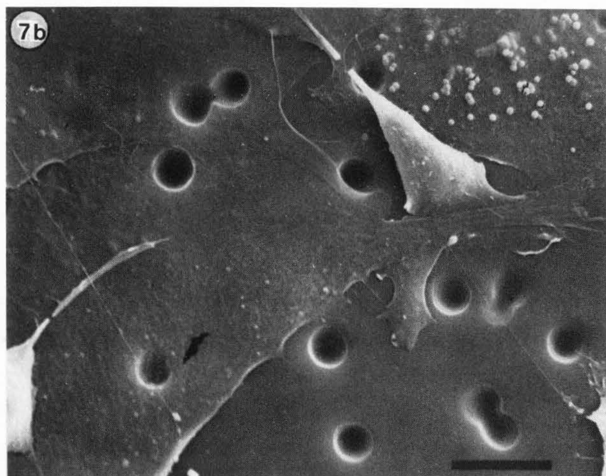


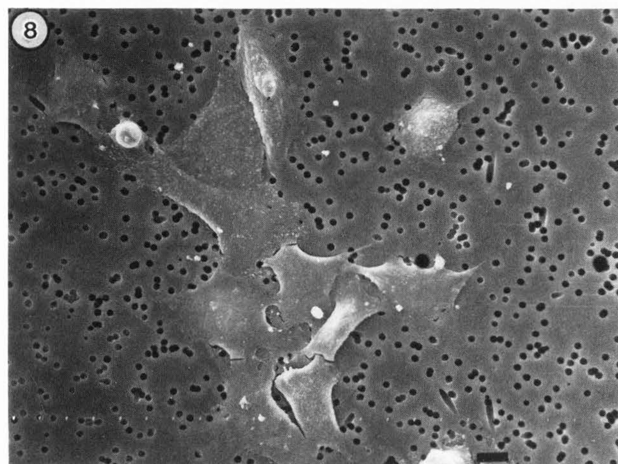


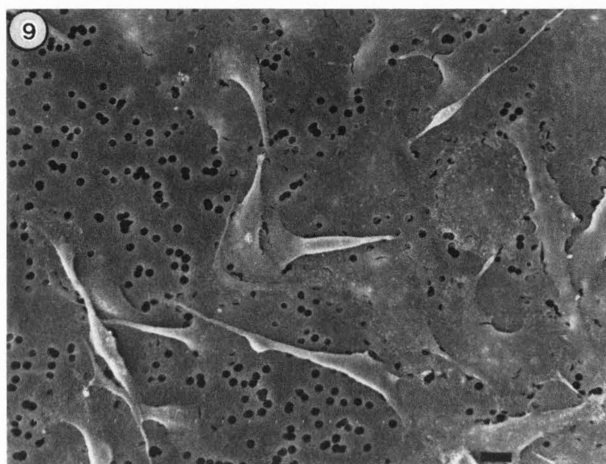












(Table 4). The number of cells per field initially suggest no significant difference in migration between untreated and BA-treated cells. However, these numbers do not take into account the partial cytostasis induced by BA. If migration rates were formulated to incorporate cellular proliferation (Fig. 1) during a three day incubation period, BA-treatment enhanced migration by 456% over controls ($p < 0.015$) (Table 5). The migration of BA-treated cells using 10% FCS was 192% greater than untreated cells using 10% calf serum ($p < 0.04$) (Trial 2, Table 4). A further enhancement of migration was induced by BA if cells were pretreated for 72 h with BA before plating. Pretreatment increased migration over non-pretreated BA-treated cells by 196% ($p < 0.005$) and similarly increased migration over untreated cells by 403% ($p < 0.02$) (Trial 2, Table 4). If 72 h migration rates were formulated to incorporate proliferation, the rate for the BA pretreated cells was 893% ($p < 0.001$) greater than untreated cells (Table 5). There was no significant difference in migration in the 18 h incubation period between untreated and BA-treated cells (Trial 4, Table 4). In contrast, the migration of RA-treated cells during the 72 h incubation period was decreased by 75% ($p < 0.03$) as compared to untreated cells, and decreased by 53% compared to cells treated with calf serum ($p < 0.005$) (Trial 2, Table 4). The level of significance between the migration rates of 10% CS-treated cells and 10% FCS-treated (CO) cells was $p < 0.1$.

Trial 3 Migration was quantified using later passage B16a cells. The methodology above was used to quantitate migration of untreated, BA-treated, and PBA-treated cells ($n = 4$). The percent migration of untreated cells was calculated to be 3.72% of the plated number. Again, we found that BA or PBA increased migration by 438% and 919%, respectively, over the untreated controls ($p < 0.03$, $p < 0.005$).

Transmission Electron Microscopy

The analysis of the cytoskeleton of untreated, RA-treated and BA-treated B16a cells was a qualitative TEM study. Analysis of untreated B16a cells found two distinct cytoskeletal phenotypes. Cells that were not visibly attached to the substrate displayed few microtubules and intermediate filaments. Microfilaments were visible only along cell margins. Cells that were attached to the substrate displayed a disorganized microtubule and intermediate filament system. Microfilaments were found in the cortical areas of the cytoplasm (Fig. 10). In contrast to untreated cells, RA and BA treatment altered the structure of the cytoskeleton. Retinoic acid-treated cells displayed parallel arrays of microtubules and intermediate filaments (Fig. 11). Less cortical

TRIAL	TIME	MEMBRANE PORE SIZE	TREATMENT	N	MAGNIFICATION	MEAN # OF CELLS	% MIGRATION	% CHANGE IN MIGRATION	SIGNIFICANCE LEVEL (PS)
#1	72 h	3 µm	Control	4	500x	0.82 ± 0.02	1.26	100	--
	72 h	3 µm	BA	4	500x	4.19 ± 0.62	6.42	+511	0.013
EARLY PASSAGE									
#2	72 h	5 µm	Control	5	1700x*	3.11 ± 0.89	55.23	100	--
	72 h	5 µm	BA	6	1700x*	3.23 ± 0.72	57.36	+104	**
	72 h	5 µm	PBA	5	1700x*	6.32 ± 0.19	74.69	+203	0.02
	72 h	5 µm	RA	5	1700x*	0.79 ± 0.15	14.03	-75	0.03
	72 h	5 µm	CS	5	1700x*	1.68 ± 0.18	29.84	-54	0.1
LATE PASSAGE									
#3	72 h	5 µm	Control	4	1700x*	0.21 ± 0.04	3.73	100	--
	72 h	5 µm	BA	4	1700x*	0.92 ± 0.17	16.34	+438	0.03
	72 h	5 µm	PBA	4	1700x*	1.93 ± 0.23	34.27	+919	0.005
EARLY PASSAGE									
#4	18 h	5 µm	Control	3	1700x*	1.07 ± 0.21	19.00	100	--
	18 h	5 µm	BA	3	1700x*	1.16 ± 0.30	20.60	108	N.S.***

* The 1700x magnification was used to delineate cell boundaries. Care was taken not to overlap between cells.
** Refer to proliferation table, Table 5.
*** N.S. = not significant.

Table 4. The migration of B16a through 3 µm and 5 µm diameter pore polycarbonate membranes. There was a significant difference in the migration through a 3 µm diameter pore membrane between untreated and BA-treated cells ($p < 0.013$) (Trial 1). In contrast, there was no significant difference between untreated and BA-treated early passage cells (Trial 2), but there was a significant difference between untreated and BA-treated late passage cells after 72 h incubation (Trial 3). Interestingly, there was a significant increase in migration of BA-pretreated early passage cells as compared to control and BA-treated cells ($p < 0.02$, $p < 0.005$ respectively, Trial 2). There was also a significant decrease in migration of RA-treated cells as compared to control and calf serum-treated cells ($p < 0.03$, $p < 0.005$ respectively). Lastly, there was no significant difference between untreated and BA-treated cells after 18 h incubation. This table includes percent migration and percent change in migration. The percent change in migration of untreated cells was considered to be 100%.

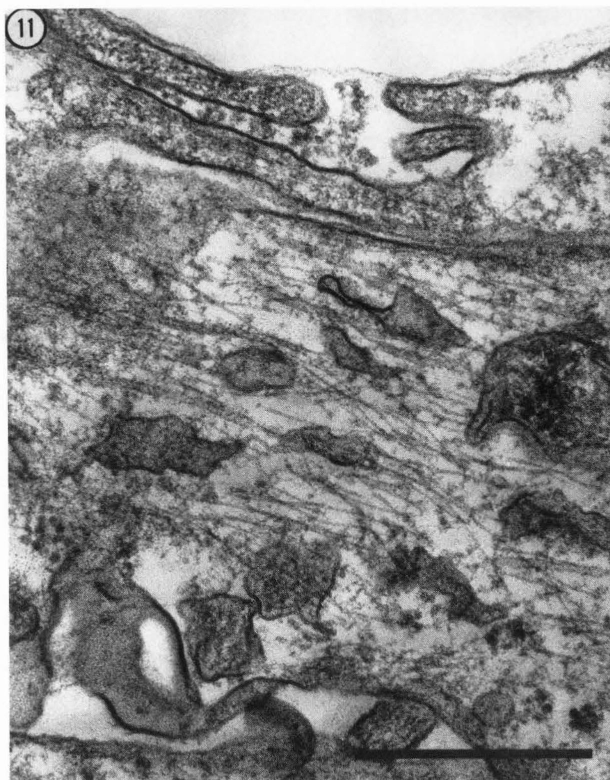
Sample Number	Control (CO)	Butyrate (BA)	Pre-butyrate (PBA)
1	0.000380	0.001830	0.001630
2	0.000122	0.000490	0.001980
3	0.000185	0.000500	0.001770
4	0.000257	0.000750	0.001740
5	0.000053	0.001008	0.001770
6		0.000873	
N	5	6	5
$\bar{X}$	0.000199	0.000908	0.001778
SD	0.000126	0.000495	0.000127
SE	0.000056	0.000020	0.000057
% mig.	100	456	893

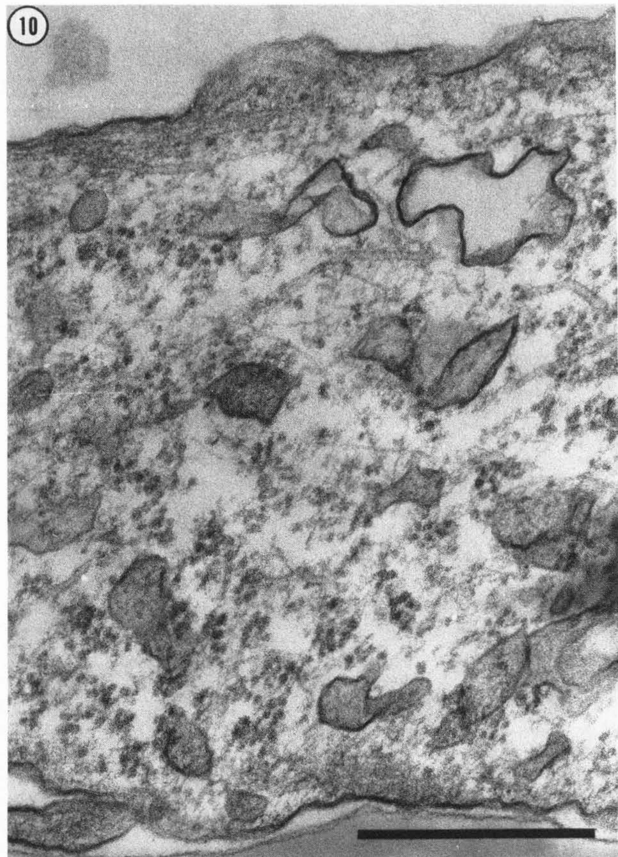
Table 5. The migration of control, BA-treated, and BA-pretreated cells after 72 h incubation with a proliferation factor incorporated into the migration rate. The migration value was defined as the number of cells per field times 50 divided by the final hemacytometer cell population after 72 h incubation (Fig. 1). There was a significant increase in migration of BA-treated and BA-pretreated cells as compared to control cells ($p < 0.015$, $p < 0.001$, respectively).

microfilaments were seen with wider focal adhesion contacts than in untreated cells. Butyric acid-treated cells also displayed parallel arrays of microtubules and intermediate filaments oriented along the longitudinal axis of the cells. An extensive cortical microfilament network was also seen (Fig. 12). This preliminary ultrastructural study supports the evidence presented in the literature that RA and BA alter the structure of the cytoskeleton.

Discussion

In this investigation, BA enhanced while RA decreased the migration of B16a cells through 3 and 5 μm diameter pores. Migration of untreated and RA-treated cells through 3 μm pore membranes was negligible (< 2% of cells plated). In contrast to untreated cells, treatment with BA resulted in a 511% increase in migration through 3 μm diameter pores. The 3 μm diameter pore is near the minimum 1 - 2 μm pore size that allows passage of B16 cells





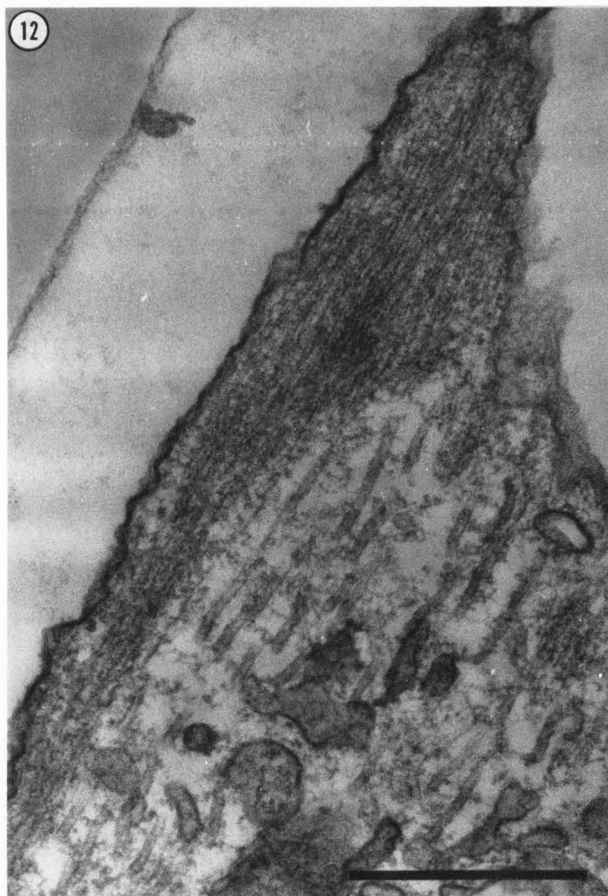


Figure 10-12. Transmission electron micrographs of untreated (Fig. 10), RA-treated (Fig. 11), and BA-treated cells (Fig. 12). The untreated cell (Fig. 10) displays a disorganized microtubule and intermediate filament system with microfilaments in the cortical area of the cytoplasm. The RA-treated cell (Fig. 11) shows an organized microtubule and intermediate filament system with less cortical actin than untreated cells. The BA-treated cell (Fig. 12) displays an extensive system of parallel microtubules and a large amount of cortical actin. (Bar = 0.5 μm).

(Tullberg and Burger, 1985). Untreated early passage (less than 6 passages) cells migrated significantly through 5 μm diameter pores (55.1%). The effect of BA on the percent change of migration through 5 μm pores was not as pronounced as through 3 μm diameter pores (104% vs 511% migration, respectively). On the other hand, RA-treated cells migrated through 5 μm pores at 14.0%, i.e., 75% less than untreated cells.

It was important to determine the cytotoxic/cytostatic concentrations of RA and BA since they vary from cell line to cell line (Reese et al., 1985; Kyritsis et al., 1984; Lotan and Nicolson, 1979). The use of non-cytotoxic drug concentrations in in vitro migration assays is important since cytotoxicity will mask enhancement or inhibition of migration (Mareel and DeMets, 1984). We used hemacytometer counts to demonstrate that RA and BA decreased proliferation during the three day incubation period. We used autoradiography and beta scintillation to show that RA and BA also inhibited the incorporation of ^{3}H-thymidine and ^{3}H-IdUR. Both RA and BA have been shown to inhibit proliferation in various tumor cell lines in the G1 phase of the cell cycle (Roberts and Sporn, 1984; Thorgeirsson et al., 1984).

In our study, phase microscopy and SEM revealed morphological changes in RA and BA-treated cells. How these changes are related to migration is not known. The untreated cell population on top of 3 and 5 μm pore membranes is heterogeneous (Figs. 4 and 5a). There are, however, more flattened cells on top of 3 μm pore membranes than 5 μm pore membranes. Concomitantly, bottom surfaces of 5 μm pore membranes are dominated by flattened cells. We conclude that increasing the pore size from 3 to 5 μm allowed more flattened cells to migrate to the bottom surface. Rounded-up cells, i.e., mitotic cells, do not migrate. It is therefore not surprising that incubation with BA, which enhances cell flattening, increases cell migration. The morphology of RA-treated cells differs from either untreated or BA-treated cells. The RA-treated cell population consisted of spindle to flattened shaped cells.

Various methods have been used to quantify motility (Volk et al., 1984; Keller and Zimmermann, 1986), deformabilty (Ochalek et al., 1988) and to correlate these properties to metastatic potential. Methods used to quantify motility include time lapse photography (Keller and Zimmermann, 1986) and the phagokinetic assay (Volk et al., 1984). Data from the above experiments have in some cases been found to be in direct conflict with data from migration assays (Spiro and Mundy, 1980). Our experimental protocol emphasizes that migration incorporates both active cellular deformabilty and motility (locomotion) (Daughaday et al., 1981). Migration is therefore defined as the net summation of cellular deformability and motility. Microtubule (MT) inhibitors, e.g., colchicine, and microfilament (MF) disrupting agents, e.g., cytochalasin B, inhibited the migration of tumor cells in a micropore filter system (Spiro and Mundy, 1980) even though cell motility was enhanced (Keller and Zimmermann, 1986). The decreased migration seen with microtubule inhibitors may be due to reduced cellular deformability (Ochalek et al., 1988). Retinoic acid is not a microtubule inhibitor, so the decrease in migration of RA-treated cells may be due to its affecting motility rather than deformability. In contrast, since the percent increase in migration of early passage BA-treated cells through the 3 μm diameter pore membrane is greater than through the 5 μm pore membrane, BA may be affecting deformability rather than motility.

Retinoic acid and BA are being investigated as potential antitumor-antimetastatic agents. Studies on various cell lines (Roberts and Sporn, 1984; Thorgeirsson et al., 1984; Ryan et al., 1987), including the B16 melanoma cell line (Lotan and Nicolson, 1979; Nordenberg et al., 1986) have stressed the effect of RA and BA on growth inhibition and differentiation. The change in morphology in these cell lines has been linked to a change in the cytoskeleton (Lehtonen et al., 1983; Ng et al., 1985; Altenburg et al., 1976; Borenfreund et al., 1980).

The role of the cytoskeleton in migration has been demonstrated by microtubule inhibitors (e.g., colchicine, vinblastine) and microfilament inhibitors (e.g., cytochalasin B) (Spiro and Mundy 1980). These inhibitors reduce migration. Both RA and BA have been shown in various tumor cell lines to increase the formation of microfilaments and vinculin plaques, and to reorganize vimentin intermediate filaments (Lehtonen et al., 1983; Ng et al., 1985; Altenburg et al., 1976; Borenfreund et al., 1980). We also observed by TEM an increase in microtubule and microfilament organization in B16a cells. Retinoic acid and BA induced changes in the cytoskeleton appear to be similar, although the surface morphology and shape of RA or BA-treated cells differ as examined by phase contrast microscopy and SEM. Subtle changes in the cytoskeleton may induce migration of BA-treated cells while inhibiting the migration of RA-treated cells. Butyric acid may have a role in increasing the amount of acetylated a-tubulin (Grant et al., 1987) thus contributing to the stabilization of microtubules (Piperno et al., 1987).

The role of cellular migration in invasion and metastasis needs further investigation. The Transwell system has merit for both migration and invasion assays. Various investigators have studied invasion by using porous polycarbonate membranes coated with a reconstituted basement membrane (Matrigel) (Albini et al., 1987; Hendrix et al., 1987; Repesh, 1989). An increase in migration of tumor cells in vitro has been linked to enhanced invasion in vitro (Grimstad, 1987; Verschueren et al., 1988). However, enhanced in vitro migration of tumor cells may not necessarily lead to increased metastasis. Grimstad (1988) found that hypermotile selected fibrosarcoma cells were less metastatic than the original parent population. Mohler et al. (1988) found, in contrast, a positive correlation between metastatic potential of Dunning R-3327 rat prostatic adenocarcinoma cells and motility. Invasive cells that form specialized pseudopodia have been interpreted by Kramer et al. (1986) to form adhesion contacts with the matrix. We observed that migratory cells (e.g., Fig. 5b) also form pseudopodia when passing through 5 μm diameter pores. In addition, we have observed by TEM focal adhesion contacts (not illustrated).

Cell motility has been linked to various parts of the cell cycle in 3T3 fibroblasts (Thurston and Palcic, 1987). Thurston and Palcic (1987) found a peak in the rate of cell movement in G2 and a significant increase in the average rate of movement as cells transit from G1 to S phase. The significance of their data, which was obtained on 3T3 cells, as applied to a tumor cell system is not known. Since

RA and BA block entry into the S phase of the cell cycle (Roberts and Sporn, 1984; Thorgeirsson et al., 1984), Thurston and Palcic's data may not be applicable. Thurston and Palcic (1987) also found a weak correlation between the rate of cell motility and the rate of cell proliferation. It is extremely important to emphasize that a decrease in proliferation is not sufficient to explain an increase in migration of BA-treated cells. A 5×10^{-5} M concentration of RA inhibits both proliferation and migration. Calf serum (CS) does not have as many of the growth factors that are in fetal calf serum. Calf serum was found to inhibit cell proliferation by 59.1% as compared to 10% FCS (Table 1). The migration of CS-treated cells is also significantly decreased from the controls ($p < 0.1$) and BA-treated cells. The migration of calf serum-treated cells, however, is significantly greater than the RA-treated cells ($P < 0.005$). Therefore, we conclude that the decrease in migration produced by RA was due to both an inhibitory effect on cell migration and a decrease in proliferation. A purely speculative explanation for the anti-migratory effect of RA is that changes in glycoproteins found on the cell surface may influence migration (Lotan et al., 1987 a,b; Couch et al., 1987, 1988).

The migration rates for untreated and BA-treated early passage cells were similar (Table 4). However, if the decrease of cellular proliferation by BA is included in defining migration, the enhancement of migration by BA is significant (Table 5). Hendrix et al. (1985) have stated that cells which invaded the amnion had similar proliferation rates as cells which did not invade. The observation that migration by preincubated BA-treated cells was significantly different from untreated and BA-treated cells suggests that further changes occur in the phenotype of these cells after 3 days. Tumor cell migration has been investigated in vitro using short time periods (4-18 h) (Varani et al., 1985; Nabeshima et al., 1986; Grimstad, 1988). It is questionable whether four hours is long enough to quantitate migration in this or any other assay. Perhaps such a short period does not adequately reflect the in vivo condition. We noted that there was no significant difference between the migration of untreated and BA-treated cells at 18 h (even if 18 h proliferation data were used in the calculations, data not shown). Cells treated for 72 h with BA before plating show a significant increase in migration (greater than both the untreated and BA-treated cells) in a three day assay (Tables 4 and 5). It would be of interest to pretreat cells with BA or RA for 72 h prior to an 18 h migration assay.

In summary, RA and BA warranted investigation for two main reasons. First, these two drugs can be less host toxic and less mutagenic than conventional chemotherapeutic drugs when administered at cytostatic concentrations. Second, the effects of RA and BA on the individual steps of the metastatic cascade are only beginning to be known.

Acknowledgements

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Discussion with Reviewers

R. Lotan: What is a diffusion chamber?
Authors: Our diffusion chamber is a modification of the Membrane Invasion Culture System (MICS) chamber (Gehlsen et al., 1984; text reference). Figure 13 is a 14 well MICS chamber. Our diffusion chamber is similar to the MICS chamber except that the diffusion chamber has 45 wells. In the migration assays, complete medium with or without a drug is added to the lower wells. The polycarbonate membrane is placed between an upper and lower chamber, creating 45 separate upper and lower wells. Cells are then added in complete medium, with or without a drug, to the upper wells.

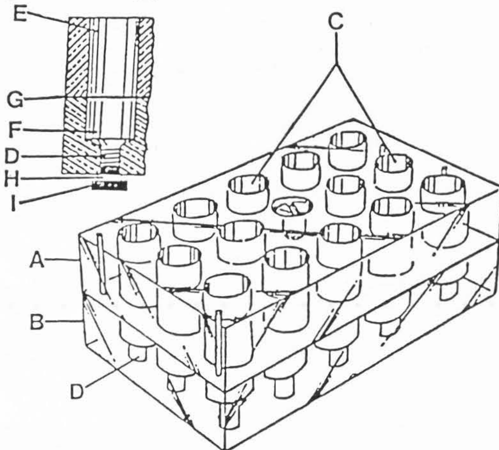

Figure 13: MEMBRANE INVASION CULTURE SYSTEM (MICS; Pat Pending 84-027-1)
(A) Top Plate; (B) Bottom Plate;
(C and E) Upper Wells; (D and F) Lower Wells;
(G) Polycarbonate Membrane;
(H) Washer; (I) Bolt.
(Redrawn with permission from Hendrix et al., 1985).

M.J.C. Hendrix: It is very difficult to derive significant statistical information from Table 4 when less than seven cells are presented?
Authors: As a purely statistical question, this is a valid criticism. Table 4 presents data obtained from 50 fields at 1700X. Data could have been presented at lower magnifications, e.g., 500X. At 500X, the exact same quantitated surface area would have provided 15 fields (1,700 divided by 500 = 3.4; 50 divided by 3.4 = 15). The mean number of cells for Table 4 would therefore be 3.4X greater. We chose to present the data at 1,700X because we could positively distinguish individual cells at higher magnification.

R.Lotan: It would have been interesting if a low metastatic variant had been used in the migration assay.
Authors: This is true, but the objectives of this project are to evaluate the effect of two drugs on a highly metastatic cell line. This project is part of an overall investigation that will evaluate the effect of RA and BA on viability, proliferation, migration, invasion, and metastasis. Since the human amnionic basement membrane (HABM) assay of Liotta et al. (New method for preparing large surfaces of intact human basement membrane for tumor invasion studies. Cancer Letters, 11: 141-152, 1980) is being used as one of the invasion assays, and since the HABM assay is only valid for highly invasive cells (Welch, DR. 1986, Discussion of the suitability, availability and requirements for in vivo and in vitro models of metastasis. In: Cancer Metastasis, Experimental and Clinical Strategies; DR Welch, BK Bhuyan, and LA Liotta (eds.). Alan R. Liss, Inc., 135), low metastatic cells were not used.

M.J.C. Hendrix: Is the rapid rate of biological degradation of retinoic acid by tumor cells similar for butyric acid? If not, what bearing would this information have on the data generated in this study?
Authors: To our knowledge the rate of biological degradation of BA by tumor cells has not been reported. The incorporation of tritiated RA by murine S91 melanoma cells has been reported to be rapid during the first 15 min. Tritiated RA is incorporated much more slowly after this initial 15 min time period (Lotan et al., Characterization of retinoic acid-induced alterations in the proliferation and differentiation of a murine and a human melanoma cell line in culture. Ann N.Y. Acad Sci 359: 150-170, 1981). In addition, S91 and B16 melanoma cells, when grown in 10^{-5} M RA for six days and then refed in RA-free medium, continued to proliferate at a reduced rate for 24-48 h. However, after 3 days of incubation in RA-free medium, the growth rate of previously RA-treated cells returned to that of the control cells (Lotan et al., Characterization of the inhibitory effects of retinoids on the vitro growth of two malignant murine melanomas. JNCI 60: 1035-1041, 1980). This reversible inhibition of growth of various tumor cell lines has been reported by others using BA (Prasad and Sinha, Effect of sodium butyrate on mammalian cells in culture: a review. In Vitro 12: 125-135, 1976). We have observed reversible changes in morphology of RA and BA-treated B16a cells after removal of the drug from the medium. Therefore, despite a rapid incorporation and rapid degradation of RA (and possibly BA) these drugs remain effective in changing morphology and inhibiting growth beyond the 72 h experimental time period.

M.J.C. Hendrix: Was scanning electron microscopy the only technique used to quantitate the tumor cells on the polycarbonate membranes? If so, what precautions were taken to insure that the entire filter was examined with SEM? Weren't the samples quite large to handle?
Authors: Our initial attempts to quantitate cells by light microscopy were unsuccessful because we were not able to distinguish cells above, in, and below the membrane. Radioisotope labelling of cells was also attempted, but difficulties were encountered in removing BA-treated cells from the polycarbonate

membranes. Even a 20 min trypsin/EDTA incubation failed to completely remove the BA-treated cells from the membrane. The entire membrane was observed by SEM. Care was taken to select 50 random fields to quantitate migration. Samples were not too large to handle. On the contrary the small size of the membranes permitted three complete Transwell membranes to be loaded at one time into the SEM.

M.J.C. Hendrix: Do the authors propose that SEM offers a universal quantitation approach for tumor cell invasion that supersedes currently available technology?
Authors: Scanning electron microscopy is an accurate and reproducible method for quantifying migration and invasion. SEM allows visualization of top and bottom membrane surfaces and permits observation of individual cells. SEM does not have the depth of field resolution problem that is associated with light microscopy. SEM is but one method to quantitate cell invasion and migration.

R. Lotan: Why is it probable that increasing the pore size from 3 μm to 5 μm allows "flattened" cells to migrate?
Authors: We have shown that the migration of early passage untreated B16a cells through 5 μm diameter pore membranes is 66% greater than the migration through 3 μm diameter pore membranes (Trials 1 and 3, Table 4). We have observed that cells on the top surface of the 3 μm diameter pore membrane are heterogeneous in shape and contain flattened cells. In contrast, the heterogeneous population of cells on top of 5 μm diameter pore membranes have few flattened cells. It appears that the majority of cells on the bottom surface of the 5 μm pore membrane are flat in shape.

R. Lotan: It is possible to dehydrate tissues for SEM with alcohol instead of acetone that would have allowed you to critical point dry the specimens instead of air drying them. Also, the membranes can be cut out of the plastic well and processed separately.
Authors: Despite the cracking produced by air drying, problems were not encountered in quantitating cells. The cracking produced by air drying the filters can be reduced by using hexamethyldisilazane (Nation et al. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. Stain Tech. 58: 347-351, 1983). The membranes were processed in Transwell chambers to facilitate ease of handling. Cutting out the membranes prior to alcohol dehydration causes the membranes to roll up.

Reviewer IV: Is the rate of proliferation the same between cells grown on polycarbonate membranes as cells grown on tissue culture treated plastic?
Authors: Yes. We have previously determined that there is no significant difference between B16a cell proliferation rates for cells grown on plastic dishes versus polycarbonate membranes. We quantified proliferation by plating cells into Transwell chambers (50,000 cells/well) and into a 24 well plate (Corning) (50,000 cells/well). Each experiment was done in triplicate and the number of cells determined after 72 h by hemacytometer count.

Reviewer IV: Why did the authors use two different radioisotopes to determine proliferation rates?
Authors: After completing the BA experiments with ^{3}H-thymidine, we changed radioisotopes to ^{3}H-IdUR. The irreversible binding property of ^{3}H-IdUR makes it a better radiolabel than ^{3}H-thymidine (Fidler, I. Metastasis: quantitative analysis of distribution and fate of tumor emboli labelled with ^{125}I-5-Iodo-2'-deoxyuridine. JNCI, 45: 773-782, 1971). Since BA had already been shown to produce a significant inhibition of ^{3}H-thymidine incorporation, we thought it would not be necessary to repeat the BA experiments using ^{3}H-IdUR.

Reviewer IV: The methodology of choice for determining cytotoxic/cytostatic effects of drugs is the double-label growth assay. Why did the authors not use this technique?
Authors: We agree that the double label growth assay is a preferred method to quantitate viability and proliferation in drug studies. Although the trypan blue exclusion method is less sensitive, it did provide cytotoxic/cytostatic data that was statistically significant.