DIMETHYL SULFOXIDE-INDUCED REVERSION OF SEVERAL FEATURES OF POLYOMA TRANSFORMED BABY HAMSTER KIDNEY CELLS (BHK-21)

Alterations in Growth and Morphology

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ABSTRACT

Cells of a polyoma virus transformed clonal line (Cl-I) of baby hamster kidney fibroblasts (BHK-21) were grown in medium containing 2 percent dimethylsulfoxide (DMSO). Unlike the untransformed BHK-21 cells, Cl-I cells adapted to replication in the presence of DMSO, and they exhibited a rapidly reversible phenotypic reversion of a number of properties characteristic of the transformed state. Restoration of density dependent growth inhibition with accumulation of cells in the G_1 phase of the cell cycle occurred and was associated with restoration of contact dependent behavior and with reversion of histological and ultrastructural features towards those which characterize untransformed cells. Concomitantly, Cl-I cells grown in 2 percent DMSO lost the ability to form colonies in semisolid medium. The data presented suggest that DMSO alters the expression of cellular functions which were altered as a result of viral transformation and which may be involved in cell tumorigenicity.

Polyoma virus-induced transformation of BHK-21 fibroblasts is associated with striking and stable alterations in many properties of these cells. These changes include contact and density dependent growth regulatory mechanisms (1), cell surface structure, cellular metabolism, antigenicity, and tumorigenicity in vivo. The precise molecular mechanisms which produce these virus-induced alterations remain unclear and the viral and cellular functions responsible for maintaining the stability of the transformed state remain unidentified.

Recently, several methods for obtaining transformed cells exhibiting reversion towards normal have been reported. Techniques include treatment with 5 fluoro-2-deoxyuridine (2), selection of variants with chromosome losses by use of biochemical markers (3), plating on glutaraldehyde-fixed normal cells (4), and treatment with dibutyryl adenosine 3′ 5′ cyclic monophosphate (5, 6). Recently, Friend et al. (7) demonstrated that low concentrations of dimethyl sulfoxide (DMSO) stimulate erythroid differentiation of murine RNA virus-induced erythroleukemia cells in vitro and alter somewhat the tumorigenicity of these cells for syngeneic mice. This report prompted us to extend earlier preliminary studies (8) of the effects of DMSO on BHK-21 fibroblasts transformed by a DNA virus, polyoma (PV).

This communication reports the induction by DMSO of rapidly reversible alterations in the density dependent behavior, ultrastructure, cell

DNA cycle traverse, and ability to form colonies in soft agar of a cloned line (Cl-I) of PV-transformed BHK-21 cells. Our observations suggest that these DMSO-induced alterations may represent phenotypic reversion of some of the features characteristic of PV-transformed cells towards a more differentiated state.

MATERIALS AND METHODS

Cells

BHK-21 cells (9) were obtained from Professor Michael Stoker. PV-transformed cells (Cl-I) were derived from a single cloned colony which grew after plating BHK-21 cells infected with the Toronto strain of PV in agar-containing Eagle's medium (10). The TD_{50} of this cell line for hamsters is $10^{3.3}$ cells and it has lost the density dependent inhibition of growth characteristic of the nontransformed parent BHK-21 cells. Although polyoma-specific nuclear T-antigen is not demonstrable in Cl-I cells by indirect immunofluorescence techniques it is present in small amount, and the presence of PV tumor-specific transplantation antigen is also demonstrable by challenge of PV-immunized hamsters with graded doses of Cl-I cells (Kisch, A. L., and Gould, I., manuscript in preparation). Repeated monitoring (11) of all cell lines employed in these studies failed to reveal detectable mycoplasma contamination.

1×10^6 cells were seeded into 30-ml plastic tissue culture flasks (Falcon Plastics, No. 3012; Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), tightly sealed, and incubated at 37°C.

Media

Cells were grown in modified Eagle's medium (ETC-10) containing four times the usual concentration of amino acids and vitamins, 10 percent tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), and 10 percent fetal calf serum (Hyland Laboratories, Los Angeles, Calif.).

The ability of cells to form colonies in semisolid medium was tested by suspending monodisperse cells in ETC-10 containing 0.3 percent Difco Bacto Agar at a temperature not exceeding 41°C. 2 ml of this suspension were then gently pipetted on to a base layer of 2 ml of ETC-10 containing 0.5 percent agar in a tissue culture flask which was sealed after gassing with 5 percent carbon dioxide. The cultures were examined 10 days later with a dissecting microscope for the presence of cell colonies. Cell aggregates consisting of approximately 16 or more cells were considered to represent colonies.

DMSO

Analytical reagent grade DMSO (Mallinckrodt Chemical Works, St. Louis, Mo.) was stored in tightly stoppered brown bottles and was added to liquid (ETC-10–DMSO) and semisolid (ETC-10–agar–DMSO) media just before use at a concentration of 2 percent (vol/vol), unless otherwise specified. For brevity, Cl-I cells grown in this concentration of DMSO are referred to as Cl-I–DMSO cells.

pH

Media were equilibrated with air at room temperature before measurement of pH using a Corning Model 12 meter (Corning Glass Works, Corning, N. Y.).

Cell Volume Measurements

Cells were removed from confluent monolayer cultures with Puck's Saline GM containing 0.5 mM/EDTA and 0.1 mg/ml crystalline trypsin (Worthington Biochemical Corp., Freehold, N. J.). The resulting cell suspension was diluted with an equal volume of complete Saline G containing 0.2 mg/ml soybean trypsin inhibitor (Worthington Biochemical Corp.) and 0.01 mg/ml DNase I (Worthington Biochemical Corp.). Cells were then suspended in Saline G, and cell volume measurements were made with the Los Alamos Scientific Laboratory (Los Alamos, N. M.) Cell Volume Spectrometer.

Light Microscopy

Phase photomicrographs of cell cultures growing in plastic tissue culture flasks were taken using a Unitron Model PH-BMIC inverted microscope (Unitron Instrument Co., Newton Highlands, Mass.). For light microscopy, cells were grown in sealed tissue culture chambers mounted on slides (Labtek Products No. 4804 Labtek Instruments Co., Westmont, Ill.), fixed and stained *in situ*, and photographed using a Zeiss photomicroscope containing a green-filtered tungsten light source.

Electron Microscopy

Growth medium was removed from cultures grown in plastic dishes and replaced with 3.0 percent glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 2 h. Preparations were post-fixed in 2.0 percent osmium tetroxide in phosphate buffer at room temperature for 1 h, rapidly dehydrated through an ethanol series, gently scraped from the substratum with a wooden policeman, and flat embedded in Epon 812. Thin sections were cut

perpendicular to the plane of embedment which facilitates orientation of cell profiles as to substratum-surface or medium-surface. Sections were mounted on uncoated grids, stained for 1 h in saturated aqueous uranyl acetate at 35°C, for 10 min in alkaline lead citrate at room temperature, and examined in an Hitachi HU-11C electron microscope.

Cell DNA Distribution Studies

Cells were prepared for analysis by flow microfluorometry (12, 13), a method which yields a distribution pattern of the DNA content of individual cells in the population, as follows: 96-h-old stationary monolayer cultures were dispersed as described above for cell volume determinations. Cells were chilled in an ice bath, resuspended in cold saline G, and an equal volume of cold saline G containing 20 percent formalin was added. After fixation in the cold for 18 h, cells were washed twice in distilled water, and hydrolyzed for 20 min at room temperature in 4 N HCl to expose and render active aldehyde radicals derived from DNA. After another wash in distilled water, the cells were stained by a modification of the acriflavine-Feulgen procedure of Culling and Vassar (14) employing 0.03 percent acriflavine. After three more washes in acid-alcohol (1 ml concentrated HCl in 100 ml 70 percent ethanol), the cells were resuspended in distilled water for analysis in the Los Alamos Flow Microfluorometer.

RESULTS

Effect of DMSO on Growth of BHK-21 and C1-I Cells

Fig. 1 A illustrates growth of BHK-21 and Cl-I cells grown in the presence or absence of 2 percent DMSO added 12 to 18 h after cells had attached to substratum. After 96 h of incubation, cultures which had received DMSO were divided and 10^6 cells were passaged into either fresh ETC-10 ("released" cultures) or fresh ETC-10–DMSO ("maintained" cultures) (Fig. 1 B). Initial addition of 2 percent DMSO to both Cl-I and BHK-21 cultures (Fig. 1 A) resulted in a delay of cell division. After 48 h, the rate of growth of the DMSO-treated Cl-I cells approximated that of untreated cells whereas the DMSO-

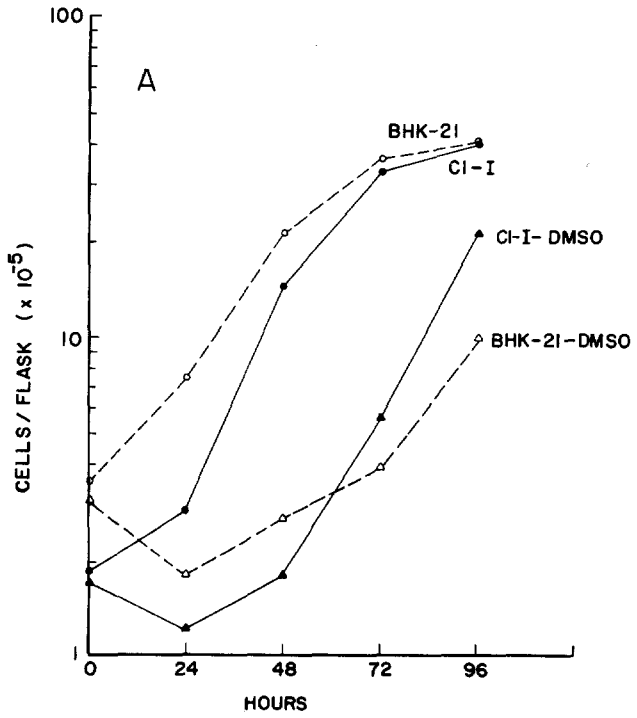


FIGURE 1 A Growth of BHK-21 and Cl-I cells in ETC-10 or ETC-10–DMSO. One million cells were seeded in ETC-10, and 2 percent DMSO was added 12–18 h after cells attached to substratum. Each point is the mean of cell counts performed on two replicate cultures.

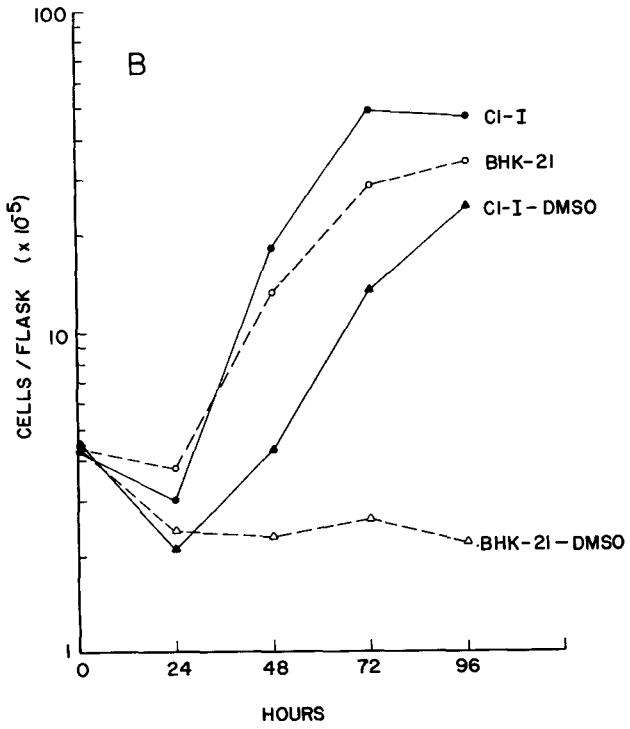


FIGURE 1 B Growth of "released" and "maintained" BHK-21 and Cl-I cells. One million cells which had been grown for 96 h in ETC-10–DMSO (Fig. 1 A) were seeded into ETC-10 or ETC-10–DMSO. Each point is the mean of cell counts performed on two replicate cultures.

treated BHK-21 cells grew more slowly. Upon release from DMSO treatment (Fig. 1 B), both cell types promptly resumed growth at a rate characteristic of untreated cells. Maintained Cl-I cells began to divide at the same time as released Cl-I cells but grew somewhat more slowly thereafter. By contrast, the growth of maintained BHK-21 cells was markedly inhibited and their number remained stationary.

These findings suggest that PV-induced transformation may confer upon BHK-21 cells the ability to adapt to replication in the presence of DMSO concentrations which progressively though reversibly inhibit the growth of the nontransformed parent line.

Light Microscope Observations

Addition of 2 percent DMSO to cultures of Cl-I cells resulted in the rapid and progressive development of striking morphological changes visible by phase microscopy and in stained preparations. The characteristic appearance of an untreated Cl-I culture 72 and 96 h after seeding is shown in Figs. 2 a and 2 b. A dense, multilayered meshwork of disoriented pleiomorphic, predominantly stellate cells is seen. Many rounded mitotic cells overlying the cell sheet are present. Prolonged incubation of such cultures beyond 4–5 days was not feasible due to rapid deterioration of the cell sheet and detachment from the glass associated with marked acidity of the medium.

By contrast, Cl-I cell sheets grown for similar periods in ETC-10–DMSO (Figs. 2 c and 2 d) exhibited typical contact-inhibited, flat, monolayered growth, and cells exhibited little tendency to pile up and form a meshwork. The individual cells are larger, more flattened, and polygonal in shape. They did not grow over each other, tending to form an ordered arrangement. Mitotic cells were present in greatly diminished numbers at 72 h and were rare to absent when the cell monolayer became confluent at 96 h. Acid production was reduced in comparison with untreated Cl-I cells (Table I). Cell cultures could be maintained in good condition with infrequent changes of

TABLE I

Organic Acid Production by Cl-I Cells

Experiment	Hours	pH of Medium*	
		ETC-10	ETC-10-DMSO
1	48	6.8	7.2
	72	6.4	6.7
	96	6.2	6.9
2	48	7.1	7.5
	72	7.0	7.5
	96	6.8	7.2

* 1×10^6 Cl-I cells were grown in ETC-10 or ETC-10 containing 2 percent DMSO. At times specified the media were harvested, equilibrated with room air, and the pH was measured.

medium for periods exceeding 3–4 wk after reaching confluence. Such prolonged maintenance of Cl-I cultures in the presence of 2 percent DMSO ultimately resulted in confluent monolayers which macroscopically resembled the typical whorled appearance of confluent BHK-21 cell monolayers. Cells became fibroblastic in appearance (Fig. 2 e), exhibiting the compact parallel orientation characteristic of BHK-21 cells (Fig. 2 f).

A striking feature of Cl-I cells grown in DMSO was their tenacious adherence to the surface on which they grew and also to each other. Removal from plastic substratum and dispersion into single cell suspension with trypsin-Versene required three to five times as long as did detachment and dispersion of comparable untreated Cl-I cultures.

In contrast to their pattern of growth in ETC-10 (Fig. 2 g), BHK-21 cells seeded in ETC-10–DMSO (Fig. 2 h) grew poorly, failed to achieve confluence, and were readily detached from the glass with 0.02% Versene and 0.025% trypsin in phosphate-buffered saline. The individual cells tended to be more delicate in appearance, exhibiting attenuated, terminal cell processes. Mitotic cells were rare to absent and acid production by such cultures was minimal.

In addition to the features already noted, nuclei of DMSO-grown Cl-I cells were larger and more ovoid than those of control Cl-I cells. Their nucleoli were somewhat reduced in number and the number of single-nucleated as well as multinucleated giant cells was substantially increased. Preparation of Cl-I–DMSO cells specifically stained to demonstrate changes in the amount or distribution of collagen or reticulin failed to reveal detectable changes compared with controls. Feulgen-stained preparations revealed Cl-I–DMSO cells to exhibit less nuclear heterochromatin, suggesting activated nucleic acid synthesis.

To determine whether DMSO-treated Cl-I cells were larger than cells grown in plain medium or merely appeared to be of increased size as a consequence of the previously noted flattening, the distribution of the volumes of cells obtained from 96-h-old cultures of Cl-I–DMSO, BHK-21, and secondary hamster embryo cells was measured. The results shown in Fig. 3 indicate that the mode of the volume of Cl-I–DMSO cells is markedly increased over that of untreated Cl-I cells. The volume distribution of Cl-I–DMSO cells more nearly resembles that of normal secondary hamster embryo cells than that of either untreated Cl-I or BHK-21 with respect to both mode and heterogeneity. The volume distribution of a population of untreated Cl-I cells incubated in ETC-10–DMSO for 15 min just before measurement was indistinguishable from that of control Cl-I cells, a finding which indicates that the increased volume of Cl-I-DMSO cells

FIGURE 2 a Cl-I cells grown in ETC-10 for 72 h. Phase photomicrograph. × 345.

FIGURE 2 b Cl-I cells grown in ETC-10 for 96 h. Hematoxylin-eosin stain. × 57.

FIGURE 2 c Cl-I cells grown in ETC-10–DMSO for 72 h. Phase photomicrograph. × 345.

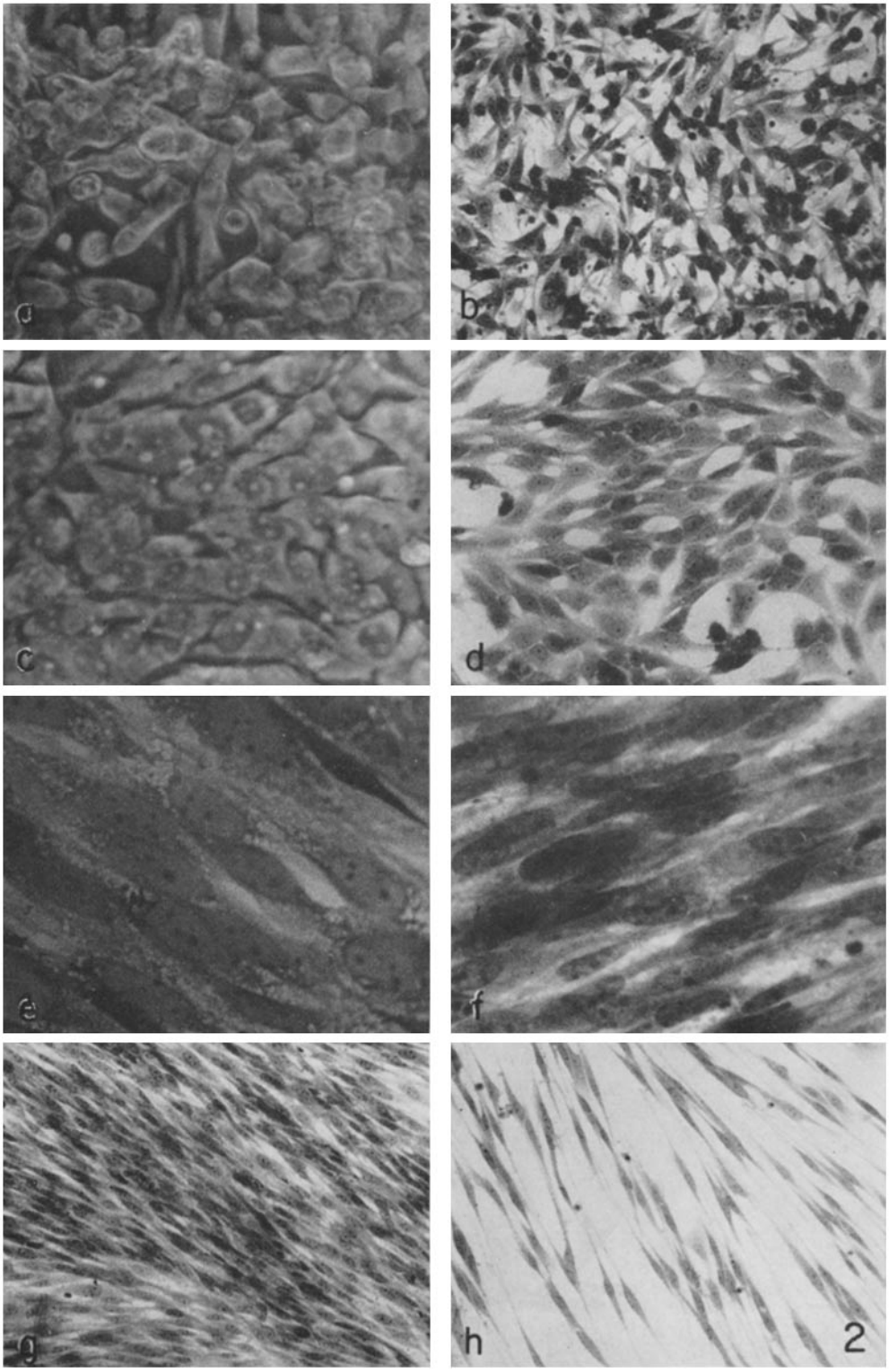
FIGURE 2 d Cl-I cells grown in ETC-10–DMSO for 96 h. Hematoxylin-eosin stain. × 57.

FIGURE 2 e Cl-I cells maintained in ETC-10–DMSO for 3 wk. Jenner Giemsa stain. × 245.

FIGURE 2 f BHK-21 cells grown in ETC-10 for 96 h. Jenner Giemsa stain. × 245.

FIGURE 2 g BHK-21 cells grown in ETC-10 for 96 h. Hematoxylin-eosin stain. × 57.

FIGURE 2 h BHK-21 cells grown in ETC-10–DMSO for 96 h. Hematoxylin-eosin stain. × 57.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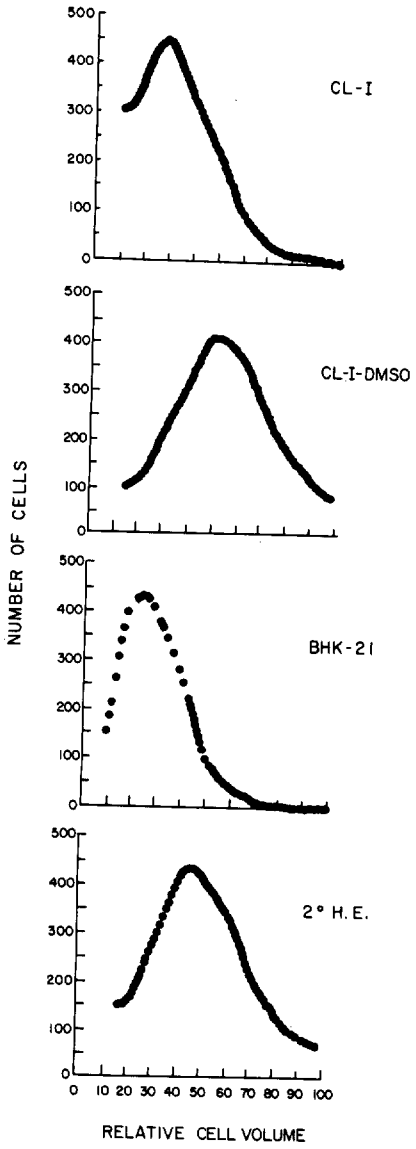


FIGURE 3 Volume distribution of cells harvested 96 h after seeding. Measurements were made with the Los Alamos Scientific Laboratory Cell Volume Spectrometer.

was not due to an osmotic effect produced by DMSO.

Early manifestations of the morphological changes described were detectable as early as 24 h after the addition of DMSO to cultures of adherent Cl-I cells. These changes were rapidly reversible upon substitution of DMSO-free ETC-10 even after prolonged maintenance or serial passage in medium containing DMSO for many weeks. Thus, resumption of non-contact-inhibited cell growth with multilayering and crisscrossing of cells, loss of flattened and fibroblastic cell morphology, decreased adhesiveness, and resumption of vigorous organic acid production again became apparent approximately 24 h after DMSO had been removed from confluent, contact-inhibited Cl-I cultures.

Concentrations of DMSO less than 2 percent produced less marked changes in the morphology of Cl-I and BHK-21 cells, and at concentrations below 1 percent such changes were not apparent. DMSO concentrations of 3 percent inhibited the growth of both Cl-I and BHK-21 cells.

Ultrastructure

The ultrastructure of Cl-I–DMSO cells was compared with that of untreated Cl-I and BHK-21 cells, with attention given to alterations which might be related to functional and structural changes induced by DMSO. Table II summarizes the major differences observed in the fine structure of the nucleus, the cytoplasm and organelles, and of the surfaces of confluent BHK-21, Cl-I, and Cl-I–DMSO cells 96 h after subculture.

As shown in Fig. 4 a, the nuclei of typical untreated BHK-21 fibroblasts are ovoid, lack indented borders, and exhibit clumps of peripheral heterochromatin adjacent to the inner leaflet of the nuclear envelope. This condensed chromatin frequently surrounds the one to two nucleoli also attached to the nuclear envelope. The body of the nucleus contains euchromatin, an abundance of 250–350 Å particles, and small tufts of condensed chromatin. Nucleoli are pleiomorphic, exhibiting both *pars amorpha* and *pars granulosa.*

The cytoplasm exhibits prominent Golgi centers, sparse granular endoplasmic reticulum (GER), numerous free ribosomes, vesicles, mitochondria, and microtubules and microfilaments (50–80 Å in diameter) oriented in the long axis of cellular processes (Fig. 4 a). Fig. 4 b illustrates a cell profile sectioned to demonstrate both the substrate (left side of micrograph) and medium surfaces (right side of micrograph). Extracellular material resembling protein polysaccharide is visible bordering the cell membrane previously attached to the culture dish, whereas dense filamentous bundles subjacent to the cell surface mark the face covered with growth medium. Microtubules and microfilaments con-

TABLE II

Summary of Ultrastructural Differences between Cell Types

	BHK	Cl-I	Cl-I-DMSO
Nucleus			
heterochromatin	+	+	−
euchromatin	+	+	+
regular border	+	−	+
indented border	−	+	−
nucleolus	+	++	+
Cytoplasm			
granular endoplasmic reticulum	−	++	+
smooth endoplasmic reticulum	−	−	−
Golgi centers	+	−	+
free ribosomes	++	+++	++
glycogen	−	−	−
oriented microtubules	++	−	++
oriented microfilaments	++	−	++
R-type virus particles	+	++	+++
Periphery			
cell-cell attachments	++	−	++
extracellular matrix (visible protein polysaccharide and collagen)	+	−	+

tinue the length of cellular processes (Fig. 4 c), the filamentous population frequently forming dense aggregates adjacent to cell surfaces. Although the distance between adjacent cells varies from 500 Å to several microns, isolated points of membrane apposition are frequently observed (arrow, Fig. 4 c).

Untreated Cl-I cells reveal rounded, less fibroblastic shapes (Fig. 5 a). Nuclei are ovoid to spherical and borders become folded and irregular. Condensed chromatin is peripheral (subjacent to the nuclear envelope) and continuous around enlarged, predominantly granular, nucleoli. Cytoplasm is densely particulate, exhibiting elaborate cisternae of granular endoplasmic reticulum containing flocculent, electron-opaque material (Figs. 5 a and 5 c). Numerous R-type virus-like particles (17–19) are visible within expanded cisternae (Figs. 5 a and 5 b) of a selected cell. Golgi centers are located in perinuclear cytoplasm, and vesicles appear limited to the Golgi zone. Fig. 5 d illustrates bundles of disoriented microfilaments in the perinuclear cytoplasm of transformed cells. These structures are localized in discrete cell regions and are lacking throughout the bulk of the cytoplasm. In contrast to BHK-21 cells, microtubules are only infrequently present.

Borders of transformed cells exhibit numerous microvilli (Fig. 5 a) which abut adjacent cells. Although structures characteristic of cell attachment (e.g. focal tight junctions and desmosomes) are sparse, cell borders are frequently seen in close association (approximately 500 Å apart). In further contrast to BHK-21 fibroblasts, Cl-I cells lack preservable (hence visible) extracellular coatings and subsurface filamentous bundles.

DMSO-treated Cl-I cells exhibit reversion to a more fibroblastic cell shape. Nuclei are ovoid, their borders are regular, and peripheral heterochromatin is present as a thin, electron-opaque zone adjacent to the inner leaflet of the nuclear envelope (Fig. 6 a). Euchromatin and 250–350 Å particles predominate in the body of the nucleus. Nucleoli (not illustrated) are similar to those present in transformed cells.

Cytoplasmic processes of DMSO-revertant cells possess numerous mitochondria surrounded by GER (Fig. 6 b) although ribosome-studded cisternae are absent in perinuclear zones. The number of R-type virus particles within expanded cisternae of rough endoplasmic reticulum is in-

creased in comparison with untreated Cl-I cells (not illustrated).

Golgi centers and vesicles are prevalent (Figs. 6 a and 6 c) in close association with cytoplasmic microtubules. Figures 6 c and 6 d illustrate microtubules and microfilaments in perinuclear cytoplasm and cell processes, respectively. Microtubules exhibit random orientation in the former, whereas microfilaments and microtubules are oriented in the long axis of the latter. Microfilaments are 50–80 Å in diameter, a larger 90–110 Å population described by McNutt et al. (20) being absent.

DMSO-treated Cl-I cells in resuming fibroblastic shape exhibit close cell-cell association, with cell membranes frequently in apposition (Figs. 6 a, 6 c, and 6 d). Fig. 6 c illustrates a focal tight junction between a slender process and the body of an adjacent cell. Micropinocytotic vesicles are visible at cell borders, which may contribute to the vesicular appearance of the perinuclear Golgi centers in revertant cells. In addition, DMSO-treated populations exhibit considerable quantities of extracellular matrix. Fig. 6 a illustrates collagen-like fibrils and material with features of protein polysaccharide between adjacent cells.

Life Cycle Distribution Analysis

The DNA distribution patterns of the cell population in confluent monolayers of secondary hamster embryo, BHK-21, Cl-I, and Cl-I–DMSO cells are shown in Fig. 7. The first (major) peak represents cells with G_1 DNA content, while at twice the mode of the G_1 DNA peak is a mode representing cells with the DNA content of cells in G_2 + M. S-phase cells with varying degrees of completion of DNA replication are distributed between these two peaks.

It is apparent that of the four populations analyzed, only Cl-I cells are actively traversing the cell cycle, with many cells present in the S and G_2 + M regions. The distribution of Cl-I–DMSO cells closely resembles the distributions which characterize confluent BHK-21 and secondary hamster embryo cell cultures. Only a small number of cells traverse S and G_2 + M, the cells being accumulated almost entirely in G_1.

These observations indicate that in the presence of 2 percent DMSO, Cl-I cells revert to a pattern of density-dependent (and possibly contact-dependent) growth inhibition characterized by an accumulation of the cells in the G_1 phase of the cell cycle. The data presented appear inconsistent with induction by DMSO of a nonspecific, toxic inhibition of growth of Cl-I cells which would result in the arrest of cells at stages throughout the cell cycle (15).

Colony Formation in Semisolid Medium

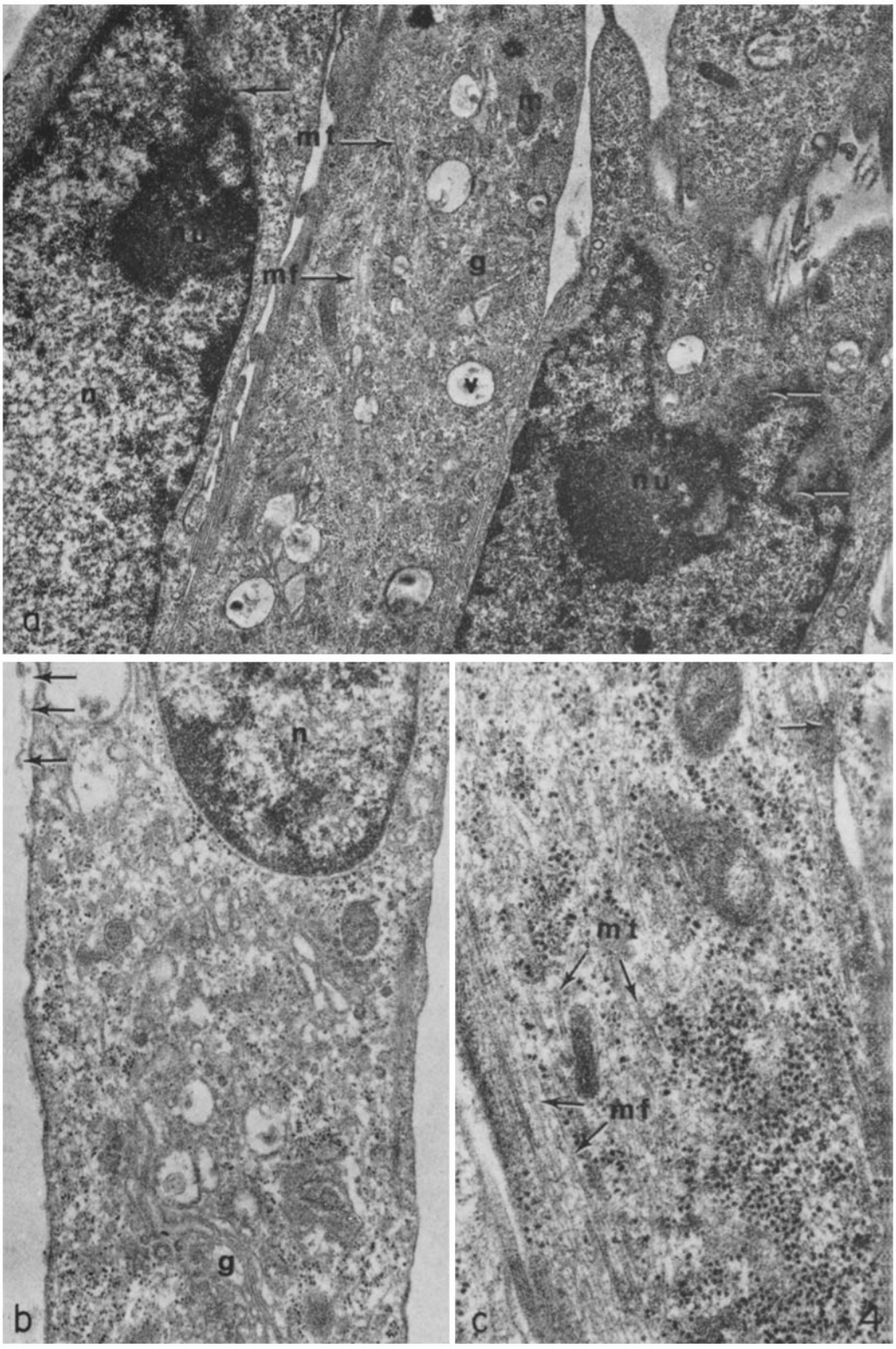
Because of rapid excretion and metabolism in the intact animal (16), the prolonged maintenance of tissue levels of DMSO comparable to those which induced and maintained the in vitro changes described herein is technically difficult, as is adequate monitoring of these levels. For this reason, the evaluation of possible DMSO-induced changes in the in vivo tumorigenicity of Cl-I cells appeared unfeasible.

Since the ability of cells to produce colonies in semisolid medium in vitro appears to be correlated with tumorigenicity in vivo (10), experiments to determine effects of DMSO on colony formation by Cl-I cells in agar-containing medium were undertaken. The results are summarized in Table III. Colony formation by Cl-I cells in ETC-10–agar–DMSO was completely inhibited whereas the cells grew readily in agar-free ETC-10 containing the same DMSO concentration. However, Cl-I cells previously maintained in fluid

FIGURE 4 a Profiles of portions of BHK-21 cells fixed *in situ*. Arrows indicate nuclear pores. *g*, Golgi center; *m*, mitochondrion; *mf*, microfilament; *mt*, microtubule; *n*, nucleus; *nu*, nucleolus; *v*, vesicle. × 12,000.

FIGURE 4 b Perinuclear cytoplasm of BHK-21 cell. Cell surface formerly attached to substratum is on the left. Arrows denote extracellular matrix with features of protein polysaccharide. *g*, Golgi center; *n*, nucleus. × 24,000.

FIGURE 4 c Longitudinal section through cell process. Note oriented microtubules (*mt*), 250 Å in diameter, and microfilaments (*mf*), 50–80 Å in diameter. Arrow indicates membrane apposition between adjacent cells. × 29,000.


mt
mf
g
nu
a
b
n
g
c
mt
mf

ETC-10–DMSO for 8 days, and exhibiting the characteristic morphological changes described above, readily formed colonies when plated in agar–ETC-10 free of DMSO.

These findings indicate that concomitant with other DMSO-induced changes, phenotypic expression of a specific property acquired by virus-transformed cells (i.e., the ability to form colonies in semisolid medium) may be reversibly altered by growing the cells in the presence of DMSO. The data presented further suggest that cell membrane functions related to tumorigenicity might similarly revert towards normal in the presence of DMSO.

DISCUSSION

DMSO is a dipolar aprotic compound whose ability to substitute for water in the hydration sheath of macromolecules (21) may be relevant to the production of some of its diverse biological effects. These have been recently summarized (22, 23, 24) and include marked changes in cell membrane permeability, nucleic acid and protein structure and synthetic processes, enzyme activity, virus-induced cell transformation (8), and cell differentiation (7).

The results reported here indicate that DMSO rapidly induces in vitro a phenotypic reversion towards nontransformed "normal" status of several properties of transformed cells believed to be controlled by integrated PV genes. However, the nearly complete inhibition of replication of BHK-21 cells and the contrasting ability of Cl-I cells to adapt to replication in medium containing 2 percent DMSO (Fig. 1 B) clearly indicate that significant deviations from the pretransformed state of the parent cell line persist in phenotypically reverted Cl-I–DMSO cells. In addition, since reversion of in vitro properties of transformed cells may be associated with increased rather than decreased tumorigenicity (25), no conclusion regarding possible alterations in this in vivo property can be drawn from our data despite the demonstration that the ability to produce colonies in agar is abolished in DMSO. It is of interest in this regard that Friend et al. (7) have demonstrated that a significant reduction in the malignancy of a cloned line of murine oncornavirus-induced erythroleukemia cells accompanied DMSO-stimulated in vitro erythroid differentiation. Whether the changes induced in Cl-I cells by DMSO described here similarly reflect an alteration in cell differentiation is at present unknown.

It would appear from the data presented that DMSO can alter the expression of transformed properties acquired as a result of transformation by polyoma, a DNA virus. However, the presence of R-type virus particles (RTP) (17, 19) in Cl-I cells and their enhancement by DMSO (18) at present makes it difficult to exclude the alternative possibility that some of the transformed properties of these cells reflect a synergistic interaction between PV and RTP which may be altered by DMSO.

The rapid reversibility of the changes produced by DMSO suggests that this compound, which is ubiquitously distributed within cells, may interfere with the availability, utilization, synthesis, degradation, or function of specific macromolecules which regulate the expression of certain transformed cell functions. Thus, the reversion of the pattern of life cycle distribution towards that characteristic of nontransformed hamster embryo cells, as indicated by the cell DNA distribution, and also the marked reduction in the amount of nuclear heterochromatin suggest that nucleic acid synthetic patterns in the transformed cells

FIGURE 5 a PV-transformed BHK-21 cell (Cl-I). Nucleus (*n*) exhibits indented borders and prominent nucleolus (*nu*); bundles of disoriented microfilaments (*mf*) and granular endoplasmic reticulum (*ger*) are apparent in the perinuclear cytoplasm. Some cisternae of the ger contain numerous R-type virus particles (arrows). × 16,000.

FIGURE 5 b R-type particles 850–950 Å in diameter, with electron-opaque nucleoids, approximately 350–450 Å in diameter, surrounded by a limiting membrane. × 74,000.

FIGURE 5 c Cisternae of granular endoplasmic reticulum in Cl-I cell containing electron-opaque, flocculent precipitate. × 14,000.

FIGURE 5 d Perinuclear cytoplasm exhibiting disoriented microfilaments (arrows). Cellular processes are absent in transformed cells. × 58,000.

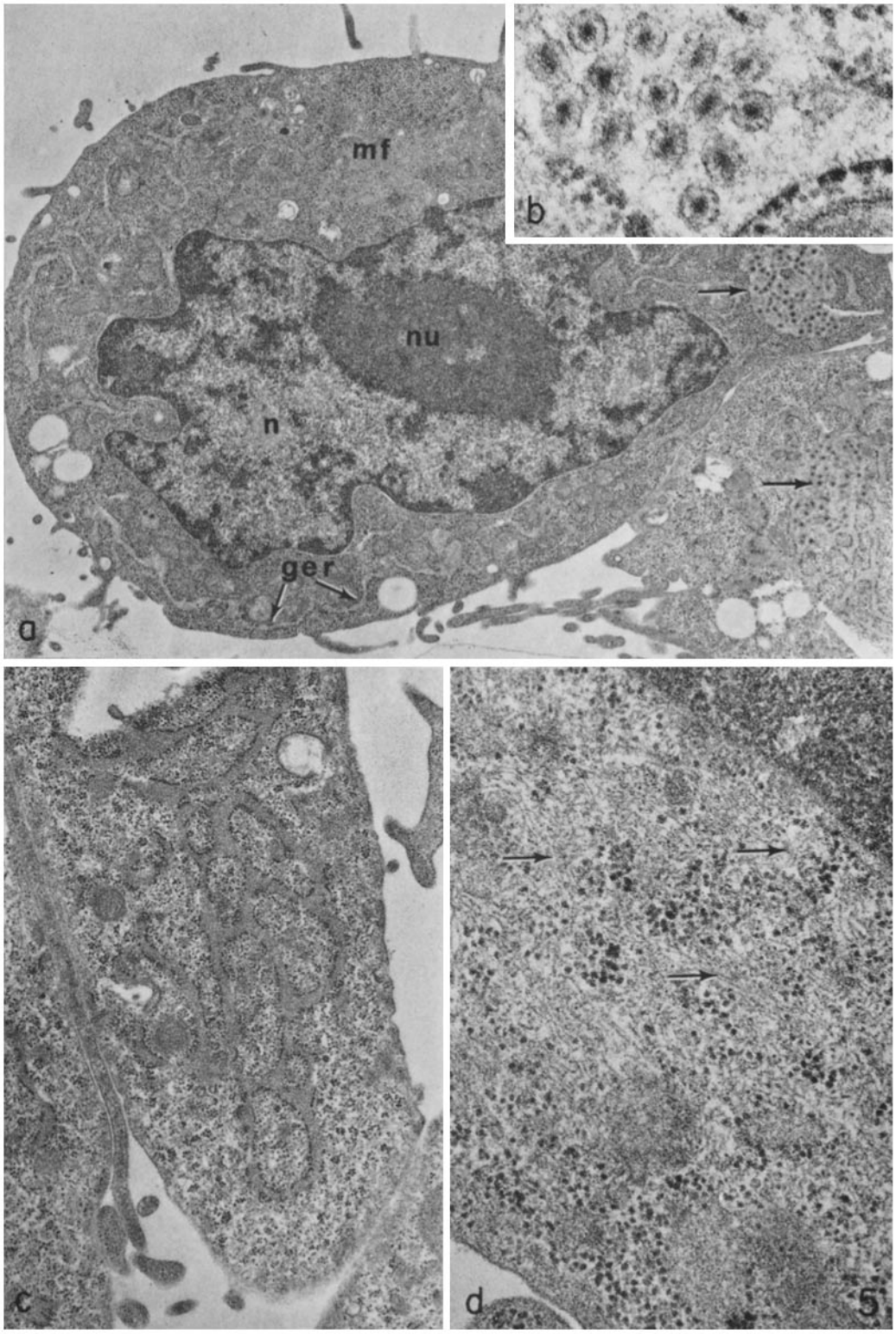

mf
nu
n
ger
a
b
c
d
5

TABLE III

Effect of 2% DMSO on Colony Formation by Cells in Agar-Containing Medium

Cell type	Colony formation in*		
	ETC-10 Agar (No DMSO)	ETC-10 Agar (2% DMSO)	ETC-10 (2% DMSO)
BHK-21	rare	0	sparse
Cl-I	TNTC‡	0	confluent
Cl-I–DMSO§	TNTC	0	confluent

* Two million cells in 2 ml of ETC-10 containing 0.3% Bacto Difco Agar with or without DMSO were inoculated on 0.5% agar–ETC-10 base in 30-ml plastic tissue culture flasks, or in 2% DMSO in agar-free ETC-10. Cultures were flushed with 5% CO_2 in air, sealed, incubated at 36.5°C for 10 days, and scored for colonies using a dissecting microscope.

‡ TNTC = colonies too numerous to count.

§ Cl-I–DMSO = Cl-I cells maintained in ETC-10, 2% DMSO for 8 days before use.

are altered by DMSO. Additionally, changes involving DNA transcription and protein synthesis are suggested by the observed ultrastructural changes in GER, ribosomes, microtubules, and microfilaments. Whether these might reflect DMSO-induced changes in cell membrane permeability (26), in the activity of such membrane-bound enzymes as adenyl cyclase (27, 28), or in intracellular concentrations of cyclic AMP (29) is at present unknown.

Although the ultrastructural basis of assuming and maintaining cell shape is obscure, microtubular proteins and other fibrous elements are believed to be involved in both cellular motility and cell process formation (30). Our data suggest that at the concentration employed, DMSO may enhance the presence of microfilaments and microtubules with an accompanying reversion of Cl-I cells towards the more fibroblastic configuration typical of untransformed BHK-21 cells. That this effect of DMSO is similar to that produced in sarcoma cells by treatment with adenosine 3′ 5′ cyclic monophosphate and its derivatives (6) again suggests the possibility that some of the effects produced by DMSO may be mediated by an effect on intracellular cyclic AMP. However, in preliminary experiments, we have found no synergism between theophylline, an inhibitor of phosphodiesterase, and DMSO with respect to the effects described here (Kisch, A. L., in progress).

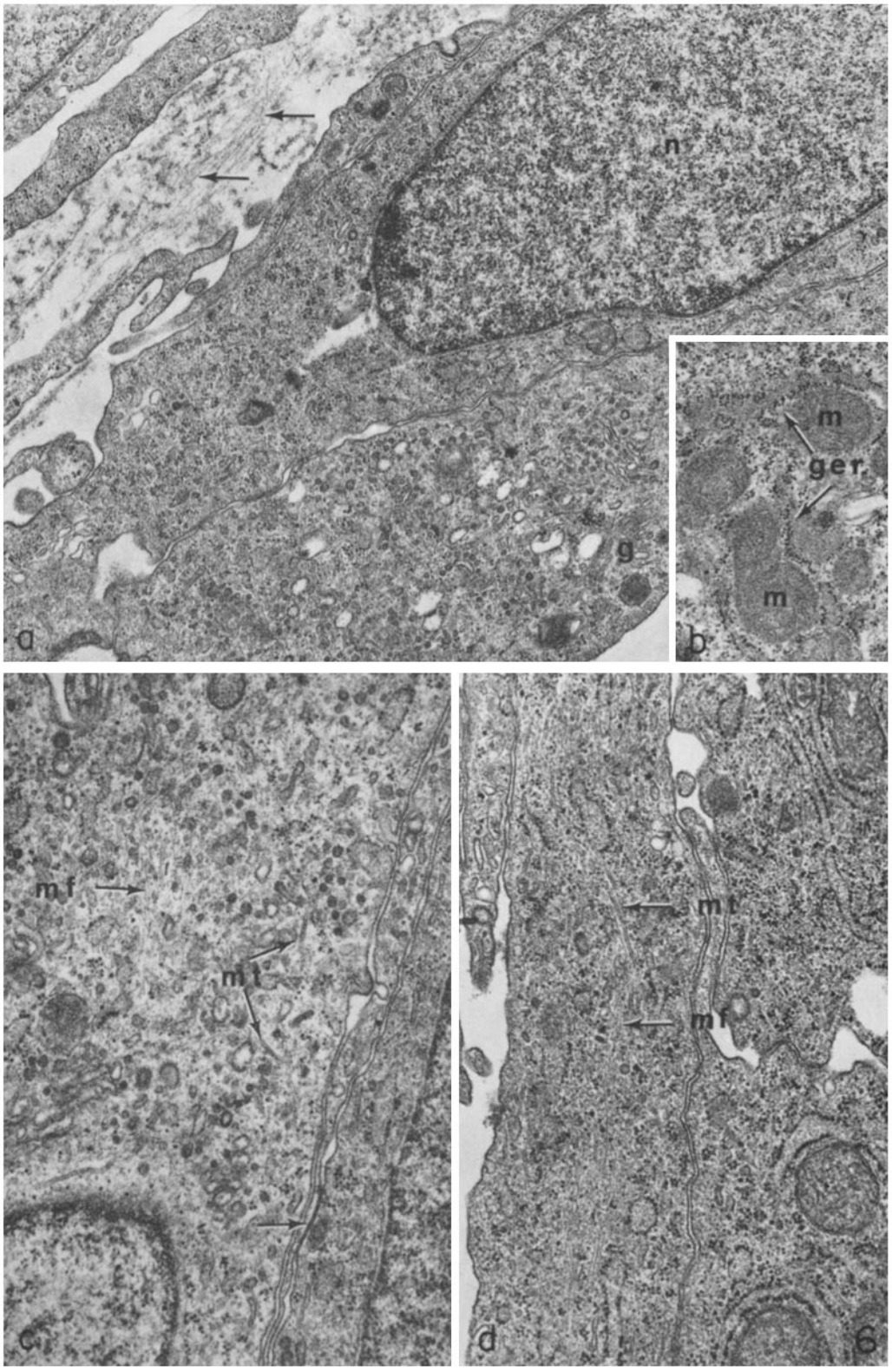
PV-induced transformation of BHK-21 cells has been shown to be accompanied by a reduction in intercellular adhesiveness (31) and by altered cell coat thickness (32) and synthetic processes (33). In the present experiments, DMSO treatment of Cl-I cells was associated with a striking restoration of the adherence of the cells both to the surface on which they grew and also to each other. While the non-contact-inhibited untreated Cl-I cells lacked demonstrable extracellular matrix and surface attachment structures such as desmosomes and tight junctions, the DMSO-treated cells exhibited the restoration of both focal tight junctions and extracellular matrix material. An inverse relationship appears to exist between cellular levels of sialic acid and both contact inhibition of growth and saturation density; contact-inhibited revertant SV3T3 clones isolated by the FUdR selection technique had regained the levels of sialic acid characteristic of nontransformed 3T3 cells (33). It would be of interest to determine whether DMSO-induced reversion of Cl-I cells to a contact inhibited growth pattern is similarly associated with an increase in the sialic acid content of the reverted cells.

FIGURE 6 a Profiles of Cl-I–DMSO cells. Nucleus (*n*) exhibits euchromatin and peripheral heterochromatin, and cytoplasm contains prominent Golgi centers (*g*). Granular endoplasmic reticulum is sparse. Arrows indicate collagen-like fibrils in a prominent, extracellular matrix. × 25,000.

FIGURE 6 b Cisternae of granular endoplasmic reticulum (*ger*) surrounding mitochondria (*m*) in Cl-I–DMSO cell process. × 49,000.

FIGURE 6 c Perinuclear cytoplasm of Cl-I–DMSO cell exhibiting microfilaments (*mf*) and microtubules (*mt*). Arrow denotes focal tight junction between cell process and body of adjacent cell. × 39,000.

FIGURE 6 d Process of Cl-I–DMSO cell with oriented microfilaments (*mf*) and microtubules (*mt*). × 24,000.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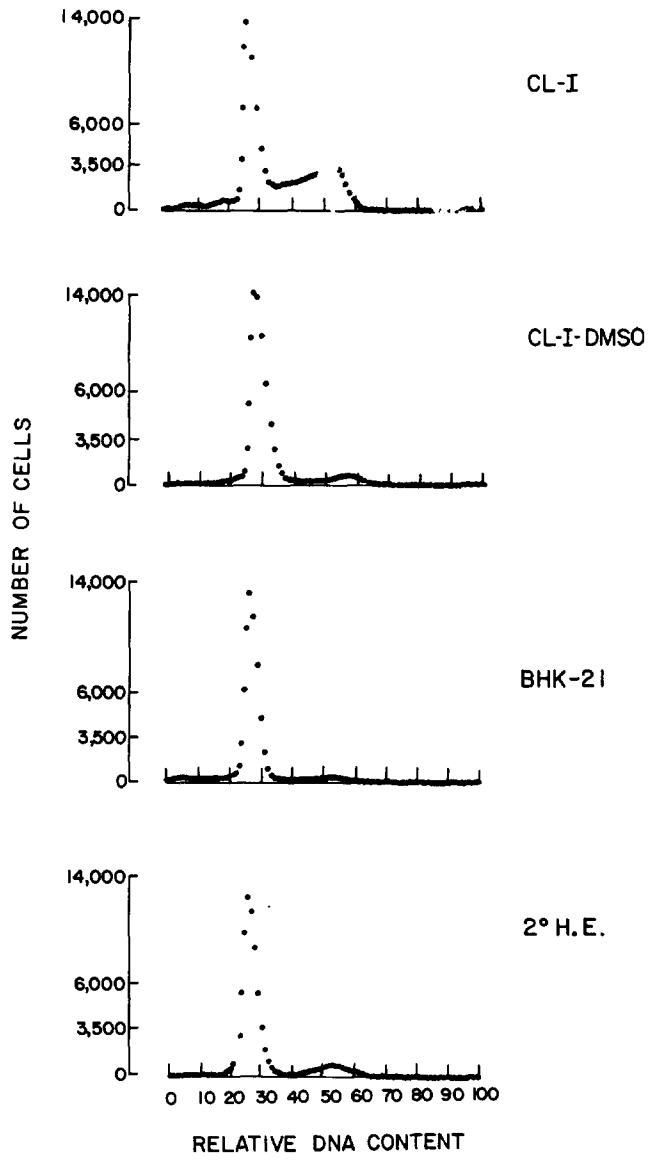

FIGURE 7 DNA distribution patterns in various cultures of cells examined with the Los Alamos Flow Microfluorometer. Cells were examined directly from confluent monolayers. DNA patterns are as follows: (*a*) Cl-I, (*b*) Cl-I–DMSO, (*c*) BHK-21, and (*d*) secondary hamster embryo. Broken lines represent modes for cells with G_1 DNA content and for cells with the DNA content of cells in G_2 + M calculated from hamster embryo. The numbers of cells examined (*a–d*) were 138,982; 83,004; 67,144; and 83,273, respectively.

Studies of the effect of DMSO on cellular collagen synthesis will be presented in a subsequent paper.

Whether reversion in the tumorigenicity of transformed cells could be achieved in vivo by administration of tolerated doses of DMSO to animals inoculated with tumor cells is at present not known, but it seems conceivable that DMSO-induced cell surface alterations specific for transformed cells might alter not only their contact dependent behavior but also the expression of antigenic specificities pertinent to immunologic rejection mechanisms.

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