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Growth- and Density-Dependent Inhibition of Deoxyglucose Transport in Balb 3T3 Cells and Its Absence in Cells Transformed by Murine Sarcoma Virus

(cell growth/cyclic AMP/low serum)

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ABSTRACT **The earliest measurable parameter that shows density-dependent inhibition is the uptake of [^{3}H]deoxyglucose by Balb 3T3 cells. The rate decreases even during the exponential phase of growth and reaches a minimum, about 8- to 10-times lower than maximum, as the culture approaches the saturation density. Cells transformed by murine sarcoma virus fail to show either growth-dependent or density-dependent inhibition of deoxyglucose uptake. Treatment with dibutyryl cyclic AMP in the presence of theophylline results in premature cessation of growth and in an arrest in the decline of deoxyglucose transport. Culture in serum-deficient medium also produces rapid inhibition of growth at low cell density, but these cultures exhibit a markedly decreased rate of deoxyglucose uptake.**

Growth *in vitro* of "normal" cells is usually well controlled in that its extent, expressed as saturation density, is limited. Several factors are thought to be involved in determining the saturation density; among them are (*i*) topoinhibition brought about by cell-to-cell proximity and (*ii*) availability of small-molecular-weight nutrients from the medium, including several factors present in the serum normally incorporated in the medium. Cells transformed by oncogenic viruses are not subject to the growth restriction brought about by crowding or lack of serum. Stoker has described the transformed malignant cells as "asocial" cells (1). Tomkins and his coworkers have considered the behavior of virus-transformed cells as analogous to the "relaxed control" of RNA synthesis in bacteria and have proposed that a "pleiotropic control mechanism," regulating several metabolic activities in eukaryotic cells, is "relaxed" in tranformed cells (2). Holley has proposed that growth of cells is controlled by modification of transport sites that regulate the availability of critical nutrients (3). The putative modifiers of the transport sites are hormones such as insulin (4), or growth factors such as the ones found in serum (5–7).

The cell-surface membrane has been implicated in the altered phenotype exhibited in growth, morphology, high lectin agglutinability, modification of glycopeptides, appearance of new antigens on the cell surface, and high hexose transport, which are considered to be characteristics of malignant transformed cells. During an investigation of increased uptake of 2-deoxy-D-glucose demonstrated by cells transformed by the Harvey strain of murine sarcoma virus (H-MSV) (8), which led to the development of a biochemical assay of the transforming activity of murine sarcoma viruses (9), we observed marked changes in the ability of *normal* cells to transport deoxyglucose. Cells transformed by H-MSV, on the other hand, fail to exhibit these changes. The data presented here indicate that the ability of cells to transport glucose is related to the extent of growth (density) of the culture and suggest to us the possibility that this relation may have growth regulatory features.

Abbreviation: H-MSV, Harvey strain of murine sarcoma virus.

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MATERIALS AND METHODS

Cells and Their Culture. Balb 3T3 cells were obtained from Dr. M. G. Sarngadharan of Litton-Bionetics Laboratories, Bethesda, Md. The cells were maintained as low-density cultures in Minimum Essential Medium supplemented with non-essential amino acids, 2 mM glutamine, 5% calf serum, and 5% fetal-calf serum. Penicillin was incorporated in the growth medium at 100 U/ml. The cells were grown in plastic tissue-culture dishes in a humidified CO_2 incubator or in 32-oz. prescription bottles at 37°. Stock cultures were stored in liquid nitrogen after resuspension in the growth medium containing 10% (v/v) dimethylsulfoxide. For growth and [^{3}H]deoxyglucose uptake experiments, cells obtained by trypsinization (0.1%) were seeded into 5-cm plastic tissue-culture dishes (Nunc). Cells were counted with an electronic particle counter (Celloscope, Particle Data, Inc., Elmhurst, Ill.).

Virus. In the experiments described here, Harvey strain of MSV was used. The virus, obtained from Dr. Henry Pinkerton of the Department of Pathology, was grown in Balb 3T3 cells. Maximum yield of virus was obtained from exponentially growing transformed cells by harvesting the culture medium at 18-hr intervals. Before use, the virus-containing fluid was filtered through a 0.22-μm membrane filter.

Measurement of 2-Deoxy-D-glucose Uptake. These assays were done essentially by the procedure described by Hatanaka *et al.* for MSV-infected mouse-embryo cells (8, 10) and by Sefton and Rubin for chick-embryo cultures (11). The following manipulations were performed at 37°. The monolayer cultures were washed with warm (37°) Hank's buffered salt solution without glucose and incubated for 10 min with 0.25 μCi of [^{3}H]deoxyglucose in 1 ml of Hank's solution −glucose. The radioactive solution was removed; cells were washed three times with ice-cold Hank's solution and then dried. The dried cells were dissolved in Lowry's C reagent (12). An aliquot was used for radioactivity determination (0.1 ml in 5 ml of toluene–methyl

cellosolve–Omnifluor, 625 ml:375 ml:9.5 g), and another aliquot appropriately diluted was used for protein determination (12).

Chemicals. Powdered tissue-culture medium and calf serum were purchased from Grand Island Biologicals, Inc., Grand Island, N.Y., and K. C. Biologicals, Lenexa, Kans. Fetal-calf serum was obtained from International Scientific Industries, Inc., Cary, Ill. Omnifluor, [^{3}H]2-deoxy-D-glucose (7.9 Ci/mmol) and [*methyl*-^{3}H]thymidine (5 Ci/mmol) were purchased from New England Nuclear and Amersham–Searle. Trypsin (1:250) was purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio, and was dissolved in 0.9% NaCl and sterilized by filtration. Theophylline and dibutyryl cAMP were from Sigma Chemical Co., St. Louis.

RESULTS

Changes in Deoxyglucose Uptake During Growth of 3T3 and MSV-Infected 3T3 Cells. During our study of the kinetics of enhancement of deoxyglucose uptake after infection of sensitive cells with H-MSV, it became apparent that the specific activity of uptake (8- to 10-fold higher than on day 0) by cells 4 days after infection was due mainly to a dramatic decrease in the specific activity of uptake by the uninfected cells (Fig. 1). Balb 3T3 cells were seeded at 3.8 × 10^5 cells per 5-cm dish with 5 ml of growth medium. After 24 hr, DEAE–dextran was added to 25 μg/ml, and the cultures were incubated at 37° for 1 hr. The medium was removed, and one set of cultures was infected with H-MSV (0.5 ml of filtered virus inoculum, about 10^4 focus-forming units per dish). During the adsorption period of 1 hr at 37°, the inoculum was redistributed every 15 min. Subsequently, 5 ml of warm growth medium was added to both the dish with infected cells and those with mock-infected cells. Uptake of [^{3}H]-deoxyglucose was measured on day 0 through day 4 after infection. The specific activity of [^{3}H]deoxyglucose uptake declined sharply in the uninfected cells after day 1, reaching a minimum 5-fold lower than the day-0 value on day 3. During the same interval, the virus-infected cells showed a slight increase in the specific activity of uptake, and on day 4, exhibited a 2-fold increase relative to the uptake on day 0. In relation to the uninfected culture on day 4, however, the specific activity was about 9-fold higher in the MSV-infected culture.

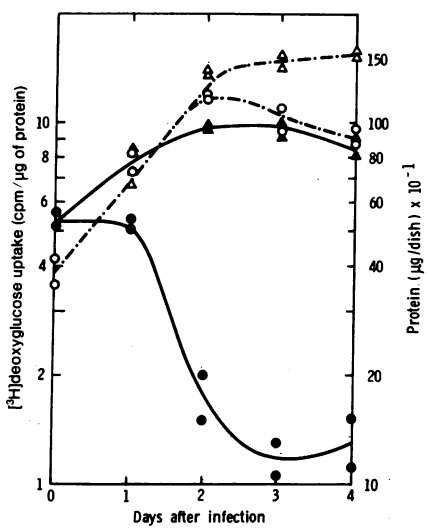

FIG. 1. Changes in deoxyglucose uptake after infection of 3T3 cells with H-MSV. 3.8 × 10^5 cells were plated per 5-cm dish. Cells were treated with DEAE–dextran and (*i*) mock-infected with medium and (*ii*) infected with H-MSV. Uptake of [^{3}H]deoxyglucose was determined in duplicate dishes with 0.25 μCi of [^{3}H]deoxyglucose (7.9 Ci/mmol) at 37° for 10 min. After removal of labeled substrate, the washed cells were dissolved in Lowry's C reagent. An aliquot was counted for [^{3}H]deoxyglucose uptake and another was used for protein determination. The rate of [^{3}H]deoxyglucose is for a 10-min incubation. Protein: O—·—O, uninfected; Δ—·—Δ, MSV-infected. [^{3}H]deoxyglucose uptake: ●——●, uninfected; ▲——▲, MSV-infected.

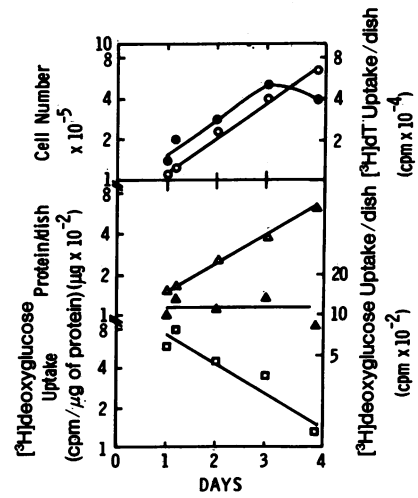

FIG. 2. Decline in deoxyglucose uptake during growth of Balb 3T3 cells. 4 × 10^5 cells per 5-cm dish were seeded on day 0. At 24-hr intervals [^{3}H]deoxyglucose and [^{3}H]dT uptake were determined. O, cells per dish; ●, [^{3}H]dT uptake during 10 min at 37° with 5 μCi of substrate; Δ, protein; ▲, [^{3}H]deoxyglucose uptake per dish per 10-min incubation; □, [^{3}H]deoxyglucose uptake, cpm/μg of protein per 10-min incubation.

The decrease in the specific activity of [^{3}H]deoxyglucose uptake is exhibited by uninfected 3T3 cells during the exponential phase of growth. The data summarized in Fig. 2 clearly show the decline in the specific activity and unchanged total uptake of [^{3}H]deoxyglucose while the cultures grow exponentially, as determined by (*i*) cell counts, (*ii*) uptake of [^{3}H]thymidine as a measure of DNA synthesis by the culture, and (*iii*) total protein per culture. These results suggest that growth of the cultures leads to a relative increase in cell density which, in turn, may result in a decrease in the hexose transport system of the cells in the culture.

Relationship of Deoxyglucose Uptake to the Culture Density of Normal and Transformed Cells. The possibility that deoxyglucose uptake reflects the potential for cell division was tested. Dishes were seeded with uninfected or H-MSV-transformed Balb 3T3 cells at increasing concentrations. We assumed that as cell number approached saturation density, cell division in the normal population would be restricted whereas cell division of transformed cells would continue. On the day after plates were seeded, cell numbers were counted and [^{3}H]deoxyglucose uptake was measured in a duplicate set of dishes. As seen in Fig. 3, [^{3}H]deoxyglucose uptake decreased as the number of normal Balb 3T3 cells increased. During a 10- to 15-fold increase in cell concentration, a reduc-

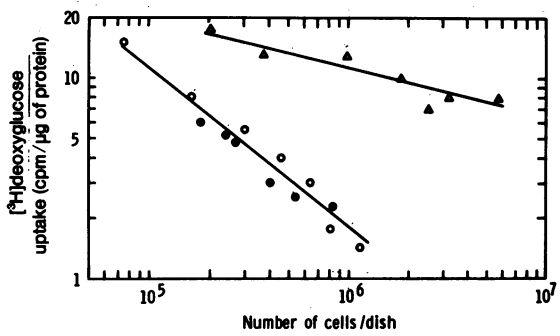

FIG. 3. Effect of culture density of uninfected (*circles*) and H-MSV-transformed (*triangles*) Balb 3T3 on deoxyglucose uptake. Different numbers of normal and transformed cells were seeded in 5-cm dishes and 24 hr later, [³H]deoxyglucose uptake was determined as described in Fig. 1. *Open* and *filled circles* are the data from two separate experiments with Balb 3T3 cells.

tion of about 8-fold in deoxyglucose uptake was observed. In contrast to this dramatic decrease in deoxyglucose uptake by normal cells, there was only about a 2-fold decrease in deoxyglucose uptake with increasing concentration of transformed cells. In other experiments, the difference has been consistently 1- to 2-fold. At 10^6 cells, the difference between deoxyglucose uptake by normal and transformed Balb 3T3 is about 10-fold.

Some Biochemical Parameters of Deoxyglucose Uptake. Since sparse cultures of 3T3 cells show high uptake of [³H]deoxyglucose, a characteristic shared by sparse as well as dense cultures of MSV-transformed 3T3 cells, we examined some biochemical features in these cells. The K_m value for uninfected 3T3 cells, measured from 0.25–20 mM [³H]deoxyglucose of constant specific radioactivity (0.2 Ci/mol), was 0.75 mM. The identical value was obtained for H-MSV-transformed 3T3 cells. The V_{max} value was 7 nmol of deoxyglucose uptake per mg of protein per min by sparse Balb 3T3 cells and by sparse or dense cultures of MSV-transformed Balb 3T3 cells (Table 1). The dependence of the rate of [³H]deoxyglucose uptake on temperature of incubation during assay and calculation of the activation energy of deoxyglucose uptake by normal and transformed 3T3 cells suggest that the systems may be qualitatively different in the two cell types (Table 1 and Fig. 4).

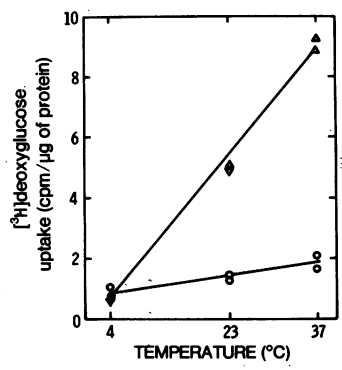

FIG. 4. Effect of temperature on the uptake of [³H]deoxyglucose by uninfected (*circles*) and H-MSV-transformed (*triangles*) Balb 3T3 cells. Uptake was measured at 4, 23, and 37° for 10 min as described in the legend to Fig. 1.

TABLE 1. *Some biochemical parameters of deoxyglucose uptake*

Parameters	Balb 3T3	H-MSV-transformed Balb 3T3
K_m	0.75 mM	0.75 mM
V_{max} (nmol/mg of protein per min)	Sparse: 7.0 Dense: 0.5–1.0	Sparse: 7.0 Dense: 4.0–7.0
Activation energy	3.6 kcal/mol	7.4 kcal/mol

Effect of Growth with cAMP and Low Concentration of Serum on Deoxyglucose Uptake. Cyclic AMP has been implicated in regulation of cellular growth. In normal cells, the intracellular level of cAMP is low during exponential phase of growth of the culture and high in nongrowing cells (13, 14). In chick fibroblasts transformed by Rous sarcoma virus, which do not show density-dependent inhibition of growth, the amount of intracellular cAMP remains low (15). There are several reports of cAMP treatment leading to a "reverse transformation," i.e., transformed cells assume normal fibroblastic morphology (16–19). The results of cAMP treatment on growth-dependent decline in deoxyglucose uptake are shown in Fig. 5. In the presence of 0.2 mM dibutyryl cAMP and 1 mM theophylline, the doubling time of Balb 3T3 is prolonged and the cells cease to grow at 5.3×10^5 per 5-cm dish. Measurement of deoxyglucose uptake in the culture treated with

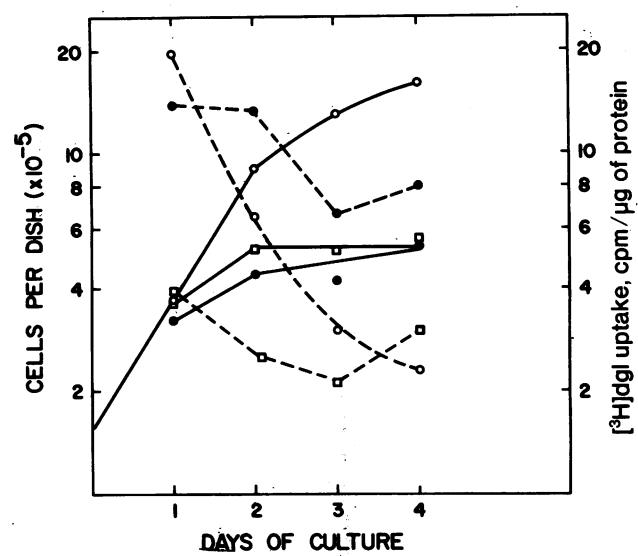

FIG. 5. Effect of growth in the presence of cAMP and low concentration of serum on deoxyglucose uptake. On day 0, 3.2×10^5 cells per 5-cm dish were seeded in (*i*) normal medium with 5% fetal and 5% calf serum; (*ii*) normal medium to which 0.2 mM dibutyryl cAMP and 1 mM theophylline were added 1 hr later; and (*iii*) medium containing only 0.5% fetal and 0.5% calf serum, which was substituted 4 hr after seeding. At 24-hr intervals, cell number and [³H]deoxyglucose uptake were determined. Cells per dish: O——O, 5% fetal + 5% calf serum; □——□, 0.5% fetal + 0.5% calf serum; ●——●, 5% fetal + 5% calf serum + 0.2 mM dibutyryl cAMP + 1 mM theophylline. Rate of [³H]-deoxyglucose uptake: O- - -O, 5% fetal + 5% calf serum; □- - -□, 0.5% fetal + 0.5% calf serum; ●- - -●, 5% fetal + 5% calf + dibutyryl cAMP + theophylline.

dibutyryl cAMP shows clearly that the decline is not nearly as marked in these nongrowing cells.

A culture was also grown in the presence of 1/10th the normal amount of serum (0.5% fetal and 0.5% calf serum). Depletion of factors present in the serum is believed to result in a premature cessation of growth without the culture becoming confluent (7). In such serum-starved sparse cultures, intracellular cAMP reaches a high level, characteristic of density-inhibited cultures (14). The rate of deoxyglucose uptake in the nonconfluent, serum-starved cultures is considerably lower even on day 1 and remains low as the culture ceases to grow by day 2. The rather dramatic difference in the rate of deoxyglucose uptake between the culture in normal medium and that in medium with a low serum concentration suggests that some serum factor(s) may play an important role in the uptake of deoxyglucose.

DISCUSSION

Competition experiments by others as well as by us (unpublished observations) with various cell lines have shown that 2-deoxyglucose enters the cell by the same transport system as glucose. The phosphorylated hexose analogue accumulates since it cannot be metabolized further. Little is known about the hexokinase system in 3T3 and transformed 3T3 cells. It would appear to play a minor role, if any, in enhancement of hexose transport seen in cells transformed by RNA tumor virus, since 3-*O*-methyl-D-glucose, an analogue that is not phosphorylated, is also taken up more rapidly by chick-embryo fibroblasts transformed by Rous sarcoma virus (20).

Density-dependent inhibition of hexose uptake by uninfected cultures of chick-embryo fibroblasts and its stimulation by serum, trypsin, neuraminidase, and insulin have been reported (4, 12). The rate of deoxyglucose uptake decreased with time after subculture of secondary cultures of chick-embryo fibroblasts (21). Treatments that provide relief of density-dependent inhibition of DNA synthesis and cell division lead very rapidly to increased uptake of deoxyglucose. Also, the stimulation is strongly dependent on protein synthesis but only weakly on RNA synthesis (11). Griffiths obtained data with a human diploid cell line showing reduced accumulation of glucose by dense culture, while a heteroploid cell line failed to show the density-dependent reduction in nutrient uptake (22). No kinetic measurements were done in this study.

Our data presented here show that uptake of deoxyglucose by untransformed Balb 3T3 cells is exquisitely sensitive to the growth-induced change in culture density. The decrease in deoxyglucose uptake is manifested by the cells growing at the maximal rate and long before the density-dependent inhibition of DNA synthesis or cell division can be detected. An uptake-inhibitory density is reached with the first cell division, and with each successive division, the ability of the cells to transport deoxyglucose decreases (Figs. 1 and 2). The apparent exponential decay of the uptake rate also suggests the possible occurrence of a random inactivation of the process.

Balb 3T3 cells that have been transformed by MSV are clearly distinguished from uninfected cells by the lack of decline of the uptake process by either growth (Fig. 1) or culture density (Fig. 3). The rate of growth of both uninfected and virus-transformed cells is identical (Fig. 1). The density-dependent and growth-dependent inhibition of the rate of deoxyglucose uptake by Balb 3T3 and by MSV-transformed Balb 3T3 cells has been observed with low (1 μM) and high (2 mM) deoxyglucose concentrations (S. K. Bose and M. VijayaLakshmi, unpublished observations). Although the apparent K_m values for deoxyglucose uptake are identical (0.75 mM) for both uninfected and MSV-transformed Balb 3T3 cells, the nature of response to temperature of incubation during assay and calculation of activation energy suggest a qualitative difference in the uptake processes in the two cell types (Table 1, Fig. 4). V_{max} determinations show that sparse normal and both dense and sparse transformed Balb 3T3 cells have identical high value (7 nmol/mg of protein per min), while dense normal cells have a 5- to 10-times lower V_{max}.

We have subjected uninfected Balb 3T3 cells to two kinds of treatment to produce nonconfluent cultures. The presence of 0.2 mM dibutyryl cAMP + 1 mM theophylline, and growth in the presence of only 0.5% fetal + 0.5% calf serum, lead to slow cell growth and a premature cessation of growth. Treatment with cAMP + theophylline was inhibitory to cell growth and led to an arrest of the decline in the rate of uptake of deoxyglucose, the nongrowing treated cells exhibiting a 4-fold higher rate compared to the control culture on day 4. Cells cultured in serum-deficient medium, on the other hand, show a drastic decrease in the rate of deoxyglucose uptake even on day 1, and the data suggest that some serum factor(s) may be involved in regulation of deoxyglucose uptake. This parameter may prove useful as an assay system for evaluation of serum factors required for growth of cells in culture.

The data indicate that cell division leads to an immediate increase in culture density and an immediate decline in the rate of hexose uptake. The decline may be the result of masking of the transport process and may be an important step in control of growth. The virus-transformed cells, in marked contrast, fail to show growth- and density-dependent inhibition of hexose transport and a consequence of this insensitive reaction may be continuous growth, characteristic of malignant cells.

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