Journal of Bioenergetics and Biomembranes, Vol. 30, No. 6, 1998

Energetic and Morphological Plasticity of C6 Glioma Cells Grown on 3-D Support; Effect of Transient Glutamine Deprivation[1]

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Received August 5, 1998; accepted November 23, 1998

The energetic metabolism of rat C6 glioma cells has been investigated as a function of the proliferative and differentiation states under three-dimensional (3-D) growing conditions on microcarrier beads. First, the transient deprivation of glutamine from the culture medium induced a marked decrease in the growth rate and a differentiation of C6 cells through the oligodendrocytic phenotype. Second, the respiratory capacity of the C6 cells during short-term subcultures with or without glutamine continuously declined as a function of the cell density, in part due to the mitochondrial content decrease. During the transition from the early exponential to the plateau growth phase in glutamine-containing medium, the oxygen consumption rate per single cell decreased concomitantly with a decrease in the glucose consumption and lactate production rates. This phenomenon led to a sixfold decrease in the total ATP production flux, without significantly affecting the cellular ATP/ADP ratio, thus indicating that some ATP-consuming processes were simultaneously suppressed during C6 proliferation. In glutamine-free medium, the cellular ATP/ADP ratio transiently increased due to growth arrest and to a reduced ATP turnover. Moreover, the results indicated that glutamine is not an essential respiratory substrate for rat C6 glioma under short-term glutamine deprivation. Worth noting was the high contribution of the mitochondrial oxidative phosphorylation toward the total ATP synthesis (about 80%), regardless of the proliferation or the differentiation status of the C6 cells.

KEY WORDS: C6 glioma; plasticity; respiration; glucose metabolism; glutamine; microcarrier.

INTRODUCTION

The study of the energetic metabolism of cancer cells has evolved considerably since Warburg discovered in 1926 that they had a respiratory deficiency and in many cases, a high glycolytic rate (Warburg *et al.*, 1926). Tumor cells had been recently characterized by a highly deviated intermediary metabolism. A major feature of fast-growing tumor cells is their elevated glycolysis rate, linked to a high rate of lactate production. Therefore, ATP production has been assumed to be more dependent on glycolysis in such cells than in nontumor cells (Pedersen, 1978). Nevertheless, the cause and significance of these characteristics remained uncertain (Argilès and Lopez-Soriano, 1990).

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5-5′-dithiobis-2-nitrobenzoic acid; FCS, fetal calf serum; $\Delta\tilde{\mu}H+$, electrochemical potential difference across the mitochondrial inner membrane; GFAP, glial fibrillary acidic protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2yll-2,5-diphenyltetrazolium) bromide; PBS, phosphate-buffered saline; PCA, perchloric acid; TET, triethyltin chloride; V_O, rate of oxygen consumption, expressed as nanomoles of atom O consumed per min and per 10^6 viable cells (nat.O/min/10^6 cells).

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0145-479X/98/1200-0565$15.00/0

Furthermore, a major consequence of such a quasiexclusive lactic acid production is that glucose carbons are no longer the major carbon source for the tricarboxylic acid cycle (TCA). L-Glutamine was, therefore, a good candidate to play this role, and, indeed, was shown to be utilized as a major respiratory fuel by fast-growing, undifferentiated tumors (Kovacevic and Morris, 1972). In fact, glutamine is essential for the growth of many types of cells in tissue culture and is involved in the major processes of cell metabolism. It is a precursor for *de novo* purine and pyrimidine biosynthesis and protein synthesis. The rapid transport of glutamine into the mitochondria, through a passive, energy-independent mechanism (Carrascosa *et al.*, 1984) and the very high activity of phosphate-dependent glutaminase in malignant cells suggest that glutamine could be a good substrate for mitochondrial respiration and, therefore, play an important role in tumor oxidative and energy metabolism. Changes in the enzyme activities required for glutamine synthesis and metabolism have also been reported in several types of tumor (Weber, 1983). Nevertheless, while a high rate of glutamine oxidation was generally observed in rapidly dividing cells, the issue of the regulation and function of this metabolic pathway in cancer is not well understood.

Glutamine has been demonstrated to play a major role in the metabolism of the central nervous system, e.g., as a carbon and nitrogen carrier between neurons and astrocytes. The appearance of a tumor in an host organism induces the generation of a significant amount of glutamine by the host tissues (Shapot, 1979; Medina *et al.*, 1992). Therefore, the understanding of the regulation and function of glutamine oxidation by malignant cells and particularly in the brain is of great physiological and pathophysiological importance. Glioma cells have provided a useful model to study glial cell properties, glial factors, and the sensitivity of glial cells to various substances and long-term subcultures (Moretto *et al.*, 1997). C6 cells also exhibit with cell passages differential enzyme expression representative of either an oligodendrocyte or an astrocyte phenotype (Parker *et al.*, 1980). With early passages, C6 cells can more easily be geared toward either astrocytic or oligodendrocyte expression, but with late passages they are more assigned to astrocytic expression (Mangoura *et al.*, 1989). They have a considerable morphological plasticity in relation to culture conditions, thus making them an excellent model to study cell differentiation.

The present work deals with the predominant energetic processes involved in C6 glioma cell growth. Particularly, we evaluated the relative contribution of glycolysis and oxidative metabolism for ATP synthesis during cell growth. The influence of glutamine deprivation in the culture medium of short-term C6 subcultures was studied in order to characterize its involvement in the oxidative metabolism of C6 cells.

MATERIALS AND METHODS

Cell Culture

C6 cells were initially plated at a density of 2.5 $\times$ 10^4 cells/cm^2 in Falcon dishes (10 cm diameter), in 10 ml Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), fungizone (0.25 mg/ml), sodium bicarbonate (2 mg/ml), and fetal calf serum (FCS) (5%). Cultures were performed for 5 days in a humidified 5% CO_2/95% air atmosphere at 37°C. The culture medium was replaced every two days. At the end of the 5-day growth period, cells were confluent (5 $\times$ 10^5 cells/cm^2). They were then seeded on gelatin microcarrier beads (Cultispher-GL, Percell Biolytica AB, Sweden) (5 $\times$ 10^6 cells per 300 mg beads and 10 ml of the same culture medium). The cells were able to enter and proliferate in the beads so that the culture could be three-dimensional. The culture was maintained for various times before experiments were performed. The first experiment was performed 12 h after seeding. For experiments in the absence of glutamine, 24 h after seeding, cells were washed twice with phosphate-buffered saline (PBS) and incubated in 10 ml glutamine-free DMEM containing 5% FCS. Cells were maintained in the absence of glutamine until experiments were performed.

Immunocytochemistry was used to check the cell population obtained under both culture conditions. Immunofluorescence experiments were done with cells cultured on 10 mm diameter glass coverslips in 24-well plates. The C6 astrocytic or oligodendrocytic phenotypes were characterized either with an anti-glial fibrillary acidic protein (anti-GFAP) antibody from rabbit (DAKO Denmark) (Bignami *et al.*, 1980; Ogawa *et al.*, 1985; Nagamatsu *et al.*, 1996; Moretto *et al.*, 1997) or with an anti-galactocerebroside antibody from rabbit (Chemicon Int., USA), respectively (Ogawa *et al.*, 1985; Nagamatsu *et al.*, 1996). Anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Boe-

hringer Mannheim) were used as secondary antibodies. Single labeling experiments were carried out according to the procedure described by Voisin *et al.* (1987, 1996). Briefly, cells grown on coverslips were fixed using a paraformaldehyde 4%–Triton X-100 0.3% solution in PBS during 30 min at room temperature. Before applying the primary antibodies and between each step, cells were washed and incubated 1 h at 4°C in PBS containing 20% horse serum. Incubation with the first anti-GFAP antibodies (1/250) and the antigalactocerebroside antibodies (1/50) were performed overnight at 4°C. The presence of the bound antibodies was revealed after incubation with FITC secondary anti-rabbit antibodies (1/100), 1 h at 37°C. Coverslips were then mounted with mowiol (Calbiochem, USA) and observed.

MTT Staining Procedure and Cell Counting

The cell distribution in the beads was visualized using the MTT [3-(4,5-dimethylthiazol-2-yll-2,5-diphenyltetrazolium) bromide] staining procedure (Sigma). For this, 400 μl of bead suspension were loaded into wells in a 24-well plate. After washing with PBS, 40 μl of MTT, dissolved in calcium and magnesium-free PBS at a final concentration of 5 mg/ml, were added to each well containing the cultured beads in PBS. After a 45 min incubation at 37°C, the dark-blue cells were observed with a phase-contrast microscope.

For cell counting, an aliquot of bead suspension was collected at the end of experiments, centrifuged, and rinsed with PBS. After centrifugation, cells were dissociated from the substratum during 3 min of bead digestion with 0.25% trypsin in Ca^{2+}–Mg^{2+}–PBS, and also mechanically with a small-bore Pasteur pipette, after stopping the enzymatic action with 20% FCS. The cell viability was estimated by using the Trypan blue dye exclusion assay.

Respiration Assays

Two hours before the experiments, the culture medium was replaced and the cells on beads were transferred in 10-ml flask containing the culture medium with or without glutamine in a 5% CO_2/95% O_2 atmosphere at 37°C. The oxygen consumption rates were continuously measured polarographically at 37°C using a Clark oxygen electrode connected to a microcomputer giving an on-line display of rate values. The respiration medium was the cell culture medium supplemented with or without glutamine, maintained in a 5% CO_2/95% O_2 atmosphere, in a final volume of 2 ml. First, the basal oxygen consumption rate was measured. Then the oxygen uptake was monitored either in the presence of the protonophoric uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (2.5 or 5 μ*M*), or in the presence of the F_1F_0- ATP synthase inhibitor, triethyltin chloride (TET)(10 to 20 μ*M*) (Rose and Aldridge, 1972). Under all conditions, single measurement did not exceed 20 min and cell viability (about 98%) was not significantly changed for incubation times up to 2 h.

Determination of Glucose and Lactate Contents of Incubation Media.

Cells on beads were rinsed twice with PBS and transferred to 3 ml fresh DMEM with or without glutamine and containing 5% FCS in all cases. They were maintained at 37°C in a 5% $CO_2$95% O_2 atmosphere. For 2 h, aliquots of the medium (175 μl) were sequentially removed every 20 min immediately added to 25 μl of a solution containing 35% (v/v) perchloric acid (PCA). Neutralization was performed by adding a solution of 2*N* KOH/0.3*M* MOPS until pH 6.5–7. Enzymatic assays including glucose oxidase and peroxidase for glucose and lactate dehydrogenase for lactate were performed according to the supplier's recommendations (Sigma).

Cell Extraction for Citrate Synthase, ATP, and ADP Assays

As for glucose and lactate assays, cells were rinsed twice with PBS, incubated with 3 ml DMEM with or without glutamine, and containing 5% FCS in all cases. Cells were maintained at 37°C in a 5% CO_2/95% O_2 atmosphere for at least 20 min. The bead suspension was then split in three aliquots for nucleotide determination, citrate synthase assay, and cell enumeration. For ATP and ADP determination, cells were precipitated in 65% (v/v) PCA containing 50 m*M* EDTA. The protein fraction was eliminated by centrifugation at 9000 × *g* for 4 min at 4°C. The supernatant was neutralized with 2*N* KOH/0.3 *M* MOPS and the precipitate eliminated by centrifugation. The ATP and ADP contents were measured as described by Aussedat

et al. (1984). Adenylic nucleotides were separated by high-performance liquid chromatography using a 5 μM ODS Spherisorb column (0.46 cm × 18 cm) at 30°C. Elution was carried out with 25 m*M* sodium pyrophosphate/pyrophosphoric acid, pH 5.75, with a flow rate of 1.2 ml min^{-1}. Absorbance was measured at 254 nm.

For citrate synthase assay and cell numbering, beads were dissolved as previously described. Cells were collected by centrifugation at 1000 × *g* for 5 min and rinsed with PBS. After centrifugation, they were lysed in 200 μl Tris-HCl 50 m*M* Triton X-100 0. 3% at pH 7.5. The citrate synthase (E.C. 4.1.3.7) activity was determined by monitoring the oxidation of coenzyme A (produced by citrate synthase activity) by 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm as a function of time (Srere, 1969). The enzyme activity was calculated using an extinction coefficient for DTNB of 13,600 M^{-1} cm^{-1} at 412 nm. One citrate synthase unit was equal to 1 μmole of DTNB reduced per minute per 10^6 cells.

RESULTS

C6 Cell Growth and Differentiation

To check the possible effect of the substratum on cell growth, C6 cells were grown for an 8-day period, either on 10-cm diameter Petri dishes or on microcarrier beads (Fig. 1). C6 cells entered their exponential growth 24 h after seeding. They reached a plateau phase at day 7, both on the beads and the plastic support, with a cell density of about 30 × 10^6 cells/Petri dish (Fig. 1). This was equivalent to 100 × 10^6 cells per g of beads when cultured with 300 mg of beads in the Petri dish. When glutamine was removed from the culture medium 24 h after seeding, the growth of the cells on beads began to slow down one day later and finally stopped; the cell number, after 6 days of culture, was much lower than that of the confluent cells (Fig. 1). Moreover, the cell number gradually decreased from day 7 of culture.

The morphology of C6 cells cultured in the presence and the absence of glutamine is shown in Fig. 2. They displayed a more epithelial phenotype with small rounded cellular bodies extending few or no processes in the presence of glutamine (Fig. 2a). In the absence of glutamine, a morphological differentiation of C6 cells was observed. These cells presented a spindle-shaped morphology, with extending thick elongated

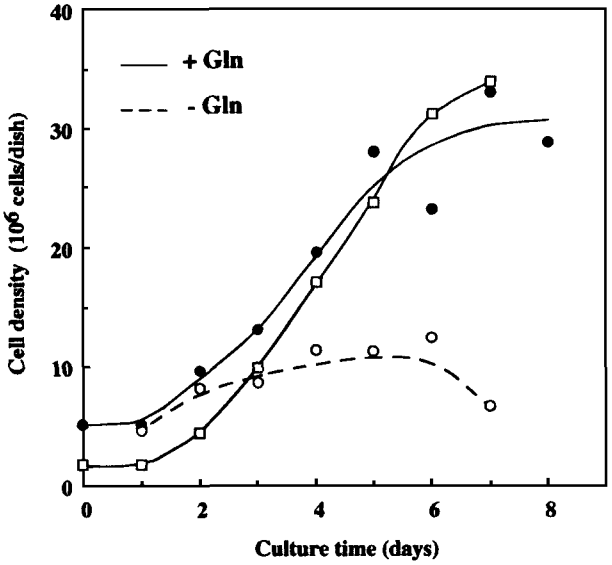

Fig. 1. Effect of culture substratum and glutamine deprivation on C6 cell growth. Growth curve (representative experiment) of C6 cells in the presence (•, □) and in the absence of glutamine (○). Cells were cultured in microcarrier beads (●, ○) or on classic Petri dish substratum (□). The culture medium was DMEM supplemented with 5% FCS and contained 4 m*M* glutamine. For cells cultured in the absence of glutamine, the deprivation of the latter from the culture medium was performed 24 h after seeding. For culture in microcarrier beads, cells were seeded at 5 × 10^6 cells for 300 mg beads and 10-ml culture medium. For culture in Petri dishes, cells were seeded at a density of 1 × 10^6 cells per 10-cm diameter dish. Whatever the cell culture condition, a lag of 24 h was observed. Cell density was expressed as viable cells per dish.

processes (Fig. 2d) in a cellular network, which was homogeneously distributed on the support. Immunocytochemistry experiments were performed to characterize the mode of differentiation of C6 cells, looking at the presence of either the astrocyte-specific glial fibrillary acidic protein (GFAP) (Bignami *et al.*, 1980) or the oligodendrocyte-specific galactocerebroside (Ogawa *et al.* 1985). In the presence of glutamine, C6 cells displayed a strong GFAP signal in the processes and the cytoplasm (Fig. 2b). Here, the intermediate filaments had a characteristic network also observable in the dividing cells (insert of Fig. 2b). In contrast, after glutamine deprivation, the GFAP labeling appeared lower and diffuse (Fig. 2e). Galactocerebroside expression appeared very different since, with glutamine, the labeling in the cytoplasm of the round cells was weak and sparsely located in the processes (Fig. 2c); in contrast, a very strong level was observed in all the cells when glutamine was discarded from the medium (Fig. 2f). These results demonstrate that the transient deprivation of glutamine from the culture

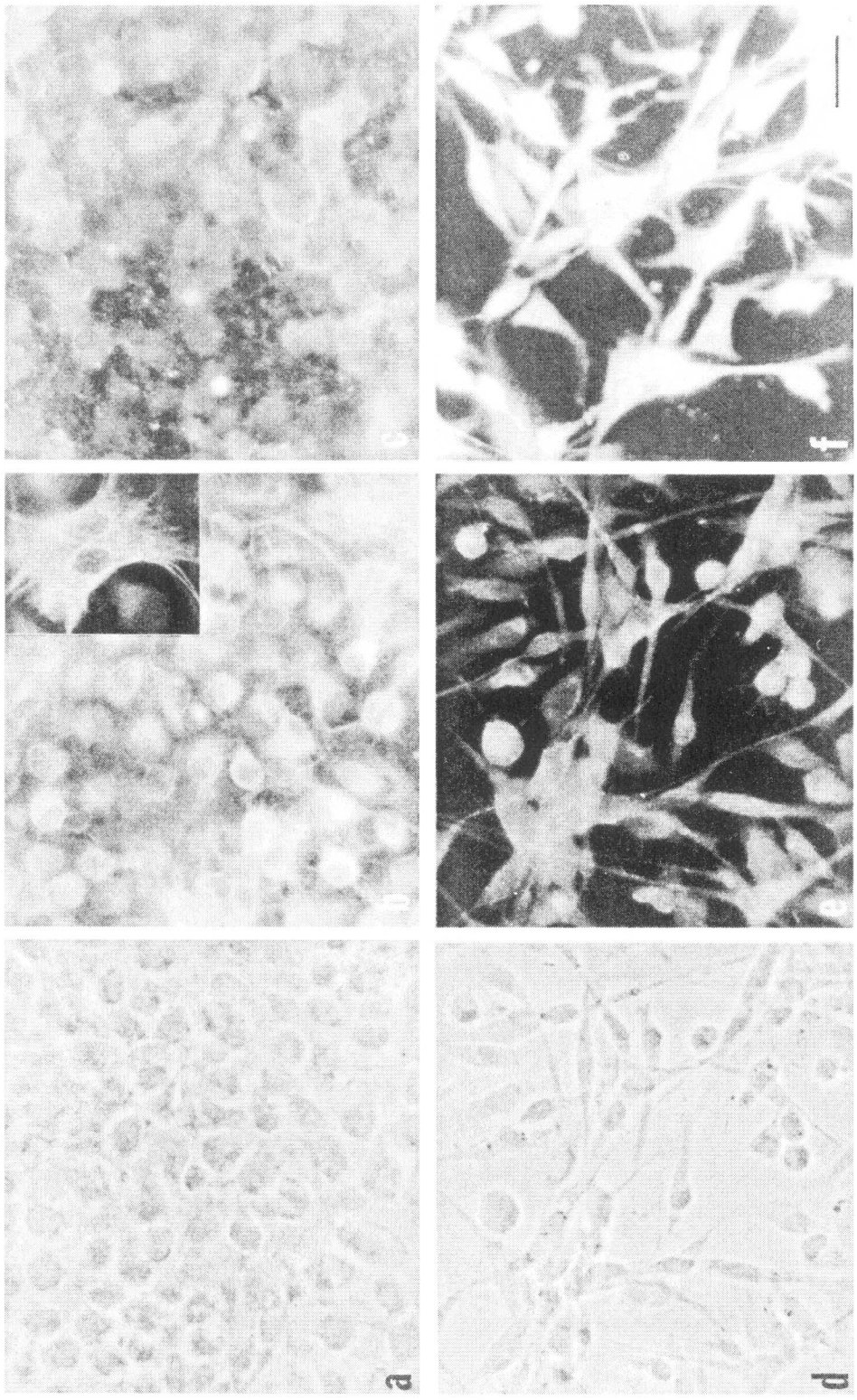

Fig. 2. Immunocharacterization of C6 phenotype. C6 cells were grown with (a, b, c) or without glutamine (d, e, f) and immunolabeled as described in Materials and Methods to characterize either the expression of the GFAP protein (b, e) or the presence of the galactocerebroside (c, f). (a) and (d) are phase-contrast pictures of the labeled fields shown respectively in (b) and (e). Scale is 25 μm. The GFAP labeling in (b) and (e), on one side, and the galactocerebroside labeling in (c) and (f), on the other, were performed with the same exposure time.

medium induced a differentiation of the C6 phenotype in the oligodendrocyte mode.

Respiratory Activity of C6 Cells

Since the aim of this work was to characterize C6 cell metabolic activities under different differentiation states, the cell culture was performed using microcarrier beads in order to minimize the morphological and functional disturbances that could occur when separating cells from their support. To compare the proliferation-associated metabolic activities of C6 cells, the measured metabolic and energetic parameters were plotted as a function of the density of viable cells instead of culture time. Figure 3A shows the evolution of oxygen consumption rates during C6 cell growth, both in the presence and in the absence of glutamine. In the presence of glutamine, for low cell density corresponding to the early exponential phase, C6 cells had a high respiratory activity (47 ± 4 nat. O/min/10^6 cells for a cell density below 5 × 10^6 cells/g of beads) (Fig. 3A). For cell density values between 5 and 25 × 10^6 cells/g of beads, respiratory activity decreased drastically, reaching a value of about 8.3 ± 2.1 nat. O/min/10^6 cells (for a cell density of about 50 × 10^6 cells/g of beads). Then, oxygen uptake remained constant in the range of cell density being in the plateau phase (6.2 ± 0.5 nat. O/min/10^6 cells) (Fig. 3A). The latter value is in accordance with measurements performed by Erecinska *et al.* (1993) on resting C6 cells (about 3 nat. O/min/10^6 cells). In our case, the large fall in oxygen consumption rate could not be correlated with a substrate depletion in the culture medium, since it was replaced every 2 days and 2 h before experiments. In the absence of glutamine, the evolution of respiratory activity of C6 cells was different. The oxygen consumption rate remained about constant at a low value (about 10 nat. O/min/10^6 cells) throughout the culture time (Fig. 3A).

Because of the difference between the respiratory activity of C6 cells in the presence and in the absence of glutamine, the maximal respiratory capacity of cells under both culture conditions was determined. For this, the oxygen consumption rates were measured in the presence of a protonophoric uncoupler, CCCP, to dissipate the proton electrochemical gradient across the inner mitochondrial membrane, and thereby to stimulate the respiratory chain activity to its maximal value (Fig. 3B). During the culture, C6 cells had the same maximal respiratory activity in the presence and in the absence of glutamine (Fig. 3B). Nevertheless, this maximal respiratory activity was also dependent on cell growth. Like basal respiratory activity (Fig. 3A), the maximal oxygen uptake decreased in the early stages of culture (from 50–60 to 10 nat. O/min/10^6 cells for a cell density between 7 × 10^6 and 20 × 10^6 cells/g of beads), and remained at a constant value

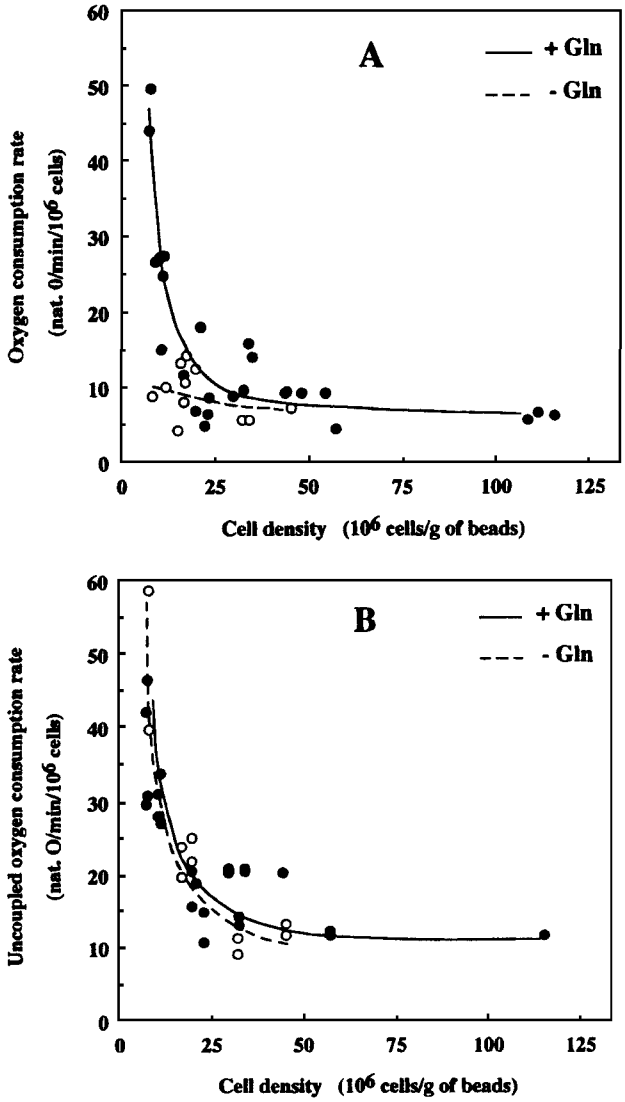

Fig. 3. Evolution of oxygen consumption rate of C6 cells as a function of cell density. (A), Basal respiratory rate of C6 cells; (B), uncoupled oxygen consumption rate of C6 cells, measured in the presence of 2.5 to 5 μ*M* of a protonophoric uncoupler, CCCP. The C6 cells were grown in microcarrier beads in the presence (•) or in the absence (○) of 4 m*M* glutamine. The oxygen uptake of the cells was measured as described in Materials and Methods and reported as a function of the number of viable cells per gram of beads (g of beads).

during the rest of the culture time (Fig. 3B). In the presence of glutamine, the basal respiratory activity of cells represented about 100% of the maximal respiratory capacity for a low cell density, whereas it was about 50% for a high cell density (cf. Fig. 3A and B). When glutamine was removed from the culture medium, the basal oxygen uptake represented only 20 to 50% of the maximum respiratory rate (cf. Fig. 3A and B), suggesting that the mitochondrial oxidative phosphorylation rate was markedly lower in the absence of glutamine.

To estimate oxygen consumption coupled to ATP synthesis for cells grown with glutamine, we used a selective inhibitor of the F_1F_0-ATP synthase inhibitor, triethyltin (TET) (Rose and Aldridge, 1972). Addition of TET induced a decrease in the oxygen uptake of C6 cells regardless of the growth phase (Table I). Moreover, during transition from early- to midexponential phase, the TET-insensitive respiration (V_O + TET) decreased from 22 to 6 nat. O/min/10^6 cells to reach a value of about 5 nat. O/min/10^6 cells in the plateau phase (Table I). This decrease throughout the culture period correlated with that of the respiratory capacity (compare V_O + CCCP with V_O + TET). Taking together the values for the basal respiratory rate of C6 cells during growth ($V_O$basal) and the O_2 uptake insensitive to TET (V_O + TET), we defined the percentage of the basal oxygen consumption coupled to ATP synthesis as: ($V_O$basal − V_O + TET)/($V_O$basal) × 100 (Table I). The calculations demonstrate that about one-half of the oxygen consumption was coupled to ATP synthesis in the early growth phase, whereas only a quarter was devoted to ATP synthesis during confluence. Finally, combining the values for the basal respiratory rate (V_O basal), the maximum respiratory capacity measured with CCCP (V_O + CCCP) and the O_2 uptake insensitive to TET (V_O + TET), we defined the percentage of stimulation of oxidative phosphorylation compared to the maximum rate as: (V_O basal-V_O + TET)/(V_O + CCCP-V_O + TET) × 100. Table I shows that the mitochondrial oxidative phosphorylation regime (i.e., ATP synthesis coupled to respiration) was maximal when cells were in an early growth phase. For the mid-exponential phase and confluence state, oxidative phosphorylation functioned at 40 and 20% of its maximal activity, respectively.

To investigate the respiratory capacity of C6 cells in relation to the mitochondrial content, we measured the specific activity of a mitochondrial enzyme (i.e., citrate synthase) as a probe of the total mitochondria content of C6 cells in the presence or in the absence of glutamine. In the presence of glutamine, the citrate synthase activity was 34% lower in the plateau phase (5–8 days of culture, 60–100 × 10^6 viable cells/g of beads) than that measured in the early exponential phase (1–2 days of culture, 1.5–10 × 10^6 cells/g of beads) (Table II). This change did not correlate with the 73% decrease in the maximum respiratory activity of the cells (Table II). Regardless of the transient glutamine deprivation (up to 7-day deprivation), the citrate synthase activity and the respiratory capacity were similar to those measured in C6 cells cultivated in the presence of glutamine for the same range of cell density (Table II).

To correlate the respiratory activity of cells with their energetic state, the ATP and ADP contents of C6

Table I. Oxidative Phosphorylation Regime of C6 Cells during Cell Growth in the Presence of Glutamine[a]

	Growth phase		
	Early exponential[b]	Midexponential[c]	Plateau[d]
V_O basal (nat. O/min/10^6 cells)	47 ± 4	8.3 ± 2.1	6.1 ± 0.5
V_O + CCCP (nat. O/min/10^6 cells)	44.2 ± 2.2	12 ± 2.5	11.7 ± 1.7
V_O + TET (nat.O/min/10^6 cells)	21.8 ± 5.4	6 ± 2	4.6 ± 0.6
(V_O basal − V_O + TET)/V_O basal × 100	54 ± 14 %	29 ± 11%	24.6 ± 3.3%
(V_O basal − V_O + TET)/(V_O + CCCP − V_O + TET) × 100	112 ± 30%	39.3 ± 17%	21 ± 4.2%

[a] The respiratory rates were measured in the growth medium without any addition (V_O basal) or either in the presence of a protonophoric uncoupler (V_O + CCCP) or a F_0F_1-ATP synthase inhibitor (V_O + TET) as described in the section Materials and Methods. Values are means ± S.D. of three to five independent measurements. (V_O basal − V_O + TET)/V_O basal × 100 represents the percentage of oxygen consumption coupled to ATP synthesis and (V_O basal − V_O + TET)/(V_O + CCCP − V_O + TET) × 100 the percentage of stimulation of oxidative phosphorylation compared to the maximum rate.

[b] 0.7–7 × 10^6 viable cells/g of beads (1–2 days of culture) for the early exponential phase.

[c] 40–60 × 10^6 viable cells/g of beads (3–5 days of culture) for midexponential phase.

[d] 100–120 × 10^6 viable cells/g of beads (5–8 days of culture) for confluence.

Table II. Citrate Synthase Activity of C6 Cells Measured after Different Culture Times in the Presence or the Absence of Glutamine in the Culture Medium[a]

	+ Glutamine		− Glutamine	
	Early exponential phase[b]	Plateau phase[b]	2-Day deprivation[c]	5- to 6-Day deprivation[a]
Citrate synthase activity (mU/10^6 cells)	23.1 ± 3.8	15.2 ± 2.3	26.6 ± 1.3	12.6 ± 3.7
Respiratory capacity (nat.O/min/10^6 cells)	44.2 ± 2.2	11.7 ± 1.3	49.2 ± 9.4	11.2 ± 1.7

[a] For comparison, the corresponding values for the mean respiratory capacity are shown. The respiratory capacity is the maximum oxygen consumption rate of C6 cells measured in the presence of the protonophoric uncoupler (CCCP). Respiratory rate and citrate synthase activity of cells were measured as described in Materials and Methods. Values are means ± S.D. of three to six independent measurements.
[b] 1.5–10 × 10^6 viable cells/g of beads (1–2 days of culture) and 60–100 × 10^6 viable cells/g of beads (5–8 days of culture) for the early exponential and the plateau phase, respectively.
[c] 15 × 10^6 viable cells/g of beads and 28–43 × 10^6 viable cells/g of beads for the 2-day and 5- to 6-day deprivation, respectively.

cells were determined for different culture times in the presence and absence of glutamine. Worth noting is that mean absolute values for cellular ATP and ADP (respectively, about 5–6 and 0.2–0.4 nmol/10^6 cells) were not significantly different from those reported in the literature for resting C6 cells using the same extraction procedure but different assays (Erecinska *et al.*, 1993). Figure 4 shows the evolution of the cellular ATP/ADP ratio calculated during cell growth. In the presence of glutamine, the ratio was not significantly altered throughout the culture period. It remained at a value of about 20 both during cell growth and at the confluence stage, thus demonstrating the stability of

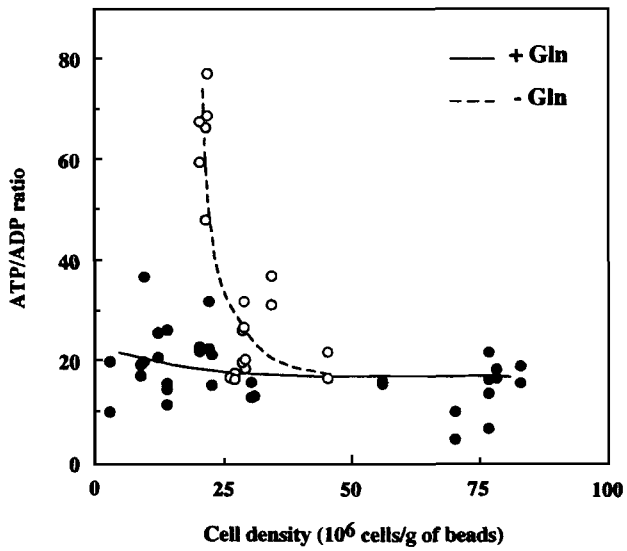

Fig. 4. Evolution of cellular ATP/ADP ratio of C6 cells as a function of cell density. The C6 cells were grown in microcarrier beads in the presence (●) or in the absence (○) of 4 m*M* glutamine. The extraction procedure and the assays of the cellular ATP and ADP content are described in Materials and Methods. Cell density was expressed as viable cells per gram of beads.

the energetic state of C6 cells during proliferation. In the absence of glutamine, the evolution of the ATP/ADP ratio was drastically different and occurred in two phases. After the transient deprivation of glutamine, when cells were in the range 25 × 10^6 cells/g of beads, there was a fourfold increase in the ATP/ADP ratio (Fig. 4). It should be noted that this rise in ATP/ADP ratio was largely due to a decrease in the cellular ADP content (data not shown). Thus, when cell densities were lower than 20 × 10^6 cells/g of beads, the ATP/ADP ratio could not be calculated since ADP determination was unreliable. When the culture aged (i.e., 5–7 days of glutamine deprivation), the ATP/ADP ratio decreased until the same value as in the presence of glutamine (about 20) was reached (Fig. 4).

Glycolytic Activity of C6 Cells

After different culture times, C6 cells were transferred to fresh medium containing initially 5.5 m*M* glucose in the presence or in the absence of glutamine. The glucose consumption and lactate production rates are reported in Fig. 5A and B. These decreased markedly during the early growth phase and remained constant during the rest of the growth period and confluence at values of about 1.6 and 3.1 nmol/min/10^6 cells for glucose consumption and lactate production, respectively. Furthermore, the ratio between the rates increased considerably for a density of cells below 17 × 10^6 cells/g of beads (Fig. 5C), evolving toward a value of 2 for a density of about 33 × 10^6 cells/g of beads and remaining constant until the end of the culture. This demonstrated that all consumed glucose molecules were converted into lactate (about 1.5 glucose for 3 lactate molecules), showing a quasiexclusive lactic fermentation during the midexponential phase and

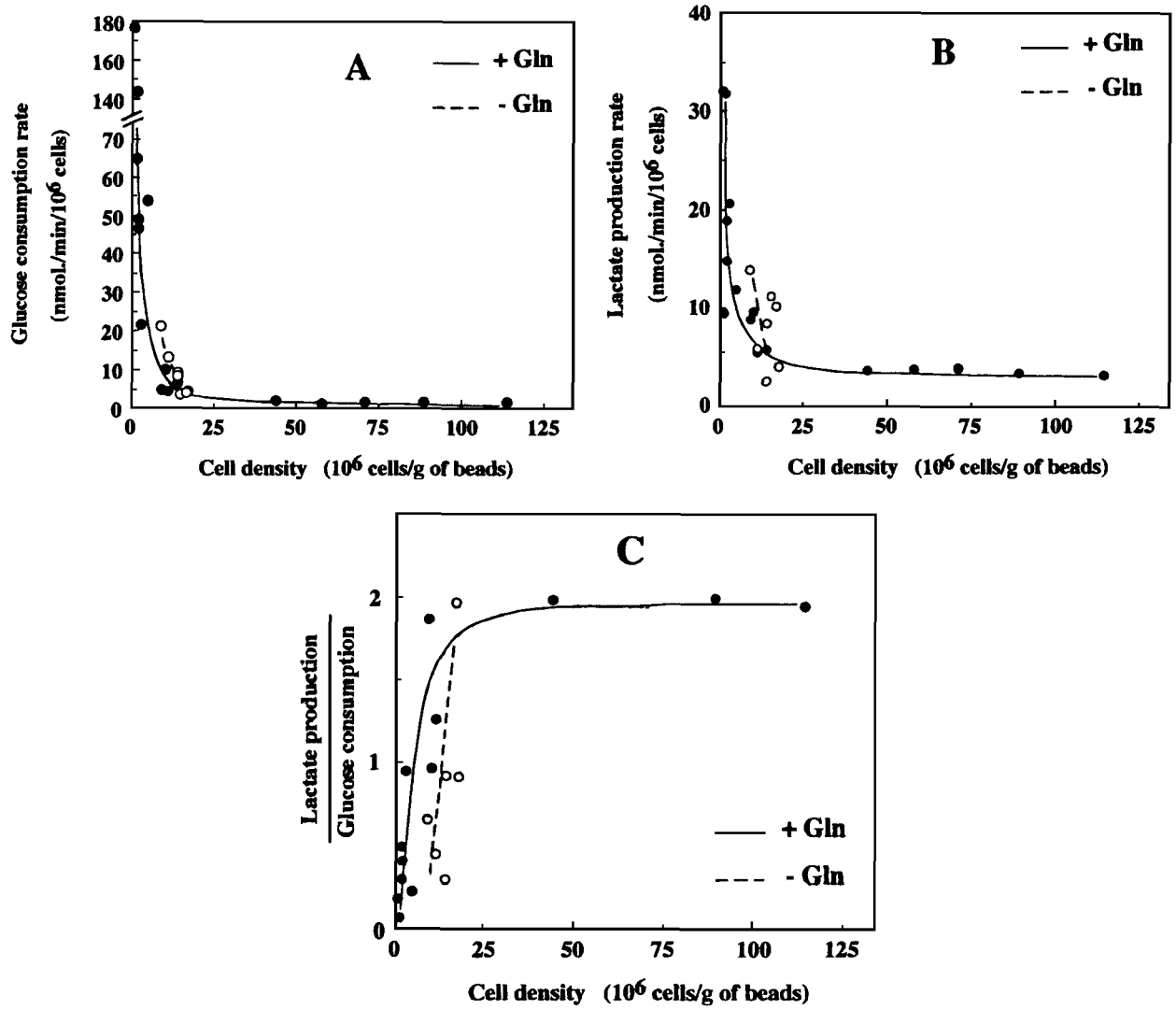

Fig. 5. Evolution of glycolytic activity of C6 cells as a function of cell density. The C6 cells were grown in microcarrier beads in the presence (●) or in the absence (○) of 4 m*M* glutamine. The glucose consumption rate (A) and the lactate production (B) were measured in the culture medium as described in Materials and Methods. From the corresponding values, the ratio of lactate production over glucose consumption was calculated (C). Cell density was expressed as viable cells per gram of beads.

at confluence. Such low glucose consumption and lactate production rates (about 0.5 and 0.9 nmol/min/10^6 cells), associated with a quasiexclusive fermentation, have been already described for C6 cells in resting medium (Keller *et al.*, 1976). However, in early exponential phase and at low cell density (i.e., about 5 × 10^6 cells/g of beads), our data showed that only 10% of glucose was converted into lactate (about 150 glucose for 30 lactate molecules). This suggested that a large part of the glucose was directed through other metabolic pathways. For the same range of cell density the glucose consumption and lactate production rates were similar both in the presence and absence of glutamine (Fig. 5A and B). Therefore, for short-term subcultures in the absence of glutamine, the ratio between glucose consumption and lactate production rates was in the same range as in the presence of glutamine (Fig. 5C).

DISCUSSION

Tumor cells are characterized by a high number of metabolic and cytologic deviations, e.g., a high glycolytic activity, a disrupted oxidative metabolism, an alteration in the content and morphology of mito-

chondria (Pedersen, 1978 for review), and also in the content of enzymes involved in certain metabolic pathways. In this work, we analyzed the energetic metabolism of a rodent tumoral nervous cell line, C6 glioma cells, under 3-D growth conditions in microcarrier beads. These cells may have considerable morphologic plasticity according to their culture conditions. On this support type, cells were able to grow under conditions inducing differentiation and also minimizing any functional perturbations during measurements.

Cell Growth and Energetic Metabolism

The respiratory rate of C6 cells cultured in microcarrier beads markedly decreased during the transition from early to late exponential phase of cell growth. A similar evolution of O_2 uptake has already been described for other cell lines cultured under various conditions (Mueller-Klieser and Sutherland, 1985; Freyer and Sutherland, 1985; Walenta *et al.*, 1989; Kallinowski *et al.*, 1989; Bredel-Geissler *et al.*, 1992). The control of cellular respiration has been a subject of constant revision over the past few decades. The chemiosmotic theory gave a framework in which the proton electrochemical gradient across the mitochondrial inner membrane (i.e. $\Delta\tilde{\mu}H^+$) acts as a coupling intermediate between the respiratory chain and the ATP synthase. In mammalian cells and tissues, the control of oxidative phosphorylation is shared among individual enzymatic steps contained between the respiratory substrate supply and the ATP utilization. In addition, a direct modulation of the $\Delta\tilde{\mu}H+$ size by H^+ cycling (e.g., uncoupling) or ion transport is a process that may also change both respiratory and ATP synthesis rates (see for reviews: Brand and Murphy, 1987; Balaban, 1990; Brown, 1992). In our study, the decrease in the respiratory activity of C6 cells during growth could be mutually and nonexclusively due to: (i) a decrease in the ATP synthesis rate throughout the culture time, as a consequence of a reduced cellular ATP demand; (ii) an alteration in the inner membrane H^+ permeability leading to an *in situ* uncoupling of the mitochondria in the early growth phase; (iii) a limitation in the reduced equivalent delivery to the respiratory chain in the late growth phase and confluence, and/or (iv) a decrease in the mitochondria content per cell during the cell growth. With respect to the hypotheses (iii) and (iv), the evolution of the respiratory capacity as well as the mitochondria content of C6 cells was investigated during growth. One interesting feature is that the respiratory capacity of C6 cells, assessed by means of a protonophoric uncoupler, declines in parallel with the basal respiratory rate during the transition from early growth to late growth-plateau phase. Simultaneously, the mitochondria content estimated upon the citrate synthase activity underwent a 34% decrease, which value fits with previous published observations (Walenta *et al.*, 1989; Bredel-Geissler *et al.*, 1992). In these reports, a 30–40% decrease in the mean number of mitochondria per cell obviously accounted for the reduction in the cellular oxygen uptake. In our case, the decrease in the mitochondrial enzyme content cannot entirely explain the four fold decrease in maximal respiratory activity of the C6 cells throughout the culture period, thus suggesting that other parameters (for instance: electron input to the respiratory chain, respiratory chain complex content) may kinetically limit the respiratory capacity at the end of the C6 growth. With respect to the mechanisms (i) and (ii) (see paragraph above) that could also reduce the basal oxygen uptake, the degree of coupling of mitochondrial ATP synthesis to respiration of growing C6 cells was assessed by comparing the basal respiratory rate with the TET-insensitive O_2 uptake on one side, and with the uncoupled respiratory rate, on the other. In the early exponential phase, the basal respiration was close to the uncoupled respiratory rate, compared to about 50% in the late growth and plateau phases. The fact that the ratio between the uncoupled and nonphosphorylating respiratory rates did not vary significantly with regard to the growth phase indicates that the respiratory chain activity is controlled by the $\Delta\tilde{\mu}H^+$ to the same extent throughout the culture period, thus making the mitochondrial uncoupling hypothesis disappear. Worth noting is that 50% of the basal respiration was coupled to ATP synthesis in the early growth phase, as compared to 25% in the subsequent phases. Taken together, the rate ratio reported in Table I demonstrated that the oxidative phosphorylation regime was optimal in the early growth phase, whereas it was only 39 and 21% of its maximal rate in the late exponential and plateau phases, respectively. This evolutionary pattern obviously accounts for the change in ATP demand for growth needs during the transition from exponential phase to confluence.

A fall in the glycolytic activity (decrease in glucose consumption and lactate production rates) was observed simultaneous with the decrease in the basal respiratory activity during C6 cell growth, demonstrating that all energy production fluxes were decreased.

The ATP production rate from glycolysis and respiration was calculated taking into account the lactate production and the basal oxygen uptake of C6 cells, combined with a theoretical ATP/O ratio of 2.6 for complete oxidation of glucose (Hinkle *et al.*, 1991; Brand *et al.*, 1993). The relative contribution of mitochondrial oxidative phosphorylation to the total ATP synthesis is rather high (between 70 and 85%) and remains constant regardless of the growth phase (Fig. 6). Worth noting is the fall in the total ATP production flux during the transition between the early exponential and the plateau phase (Fig. 6). However, the effective ATP/O ratio in cells and tissues has been reported to depend on the nature of the respiratory substrate and to decrease as the oxygen consumption rate decreased (Muller *et al.*, 1986; Brand *et al.*, 1993). Consequently, the use of the ATP/O value of 2.6 in Fig. 6 may overestimate the percentage of the respiration-linked ATP synthesis to the total ATP synthesis of confluent C6 cells and may minimize the difference in ATP turnover between early exponential and confluent cells.

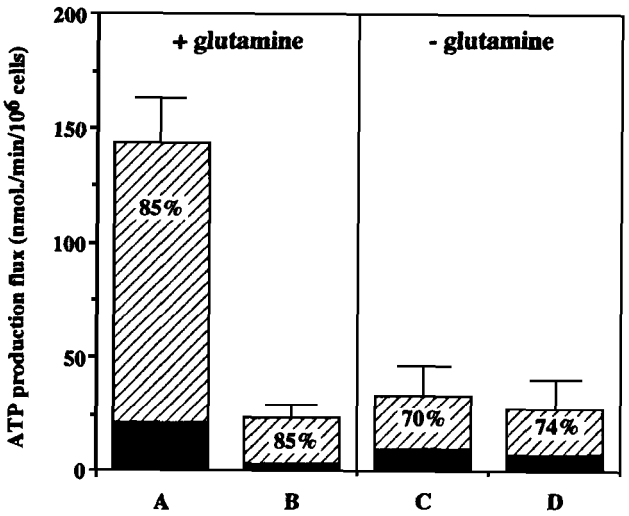

Fig. 6. Calculation of ATP-production fluxes originating from mitochondrial oxidative phosphorylation and from glycolysis of C6 cells. Conditions are as follows: (A) Early exponential phase (1–2 days of culture, 0.7–7 × 10^6 viable cells/g of beads); (B) plateau phase (4–7 days of culture, 40–120 × 10^6 viable cells/g of beads); (C) 2-day glutamine deprivation (7–14 × 10^6 viable cells/g of beads); (D) 4–7-day glutamine deprivation (28–50 × 10^6 viable cells/g of beads). Glycolytic ATP synthesis (dark-hatched boxes) was calculated from the lactate production rate (Fig. 5B). ATP synthesis due to oxidative phosphorylation (light-hatched boxes) was estimated from the oxygen uptake (Fig. 3A) and assuming an ATP/O ratio of 2.6 (Hinkle *et. al.*, 1991). The percentage values in light-hatched boxes represent the relative contribution of the oxidative phosphorylation to the total ATP-production flux.

Under steady state conditions, where ATP synthesis must match ATP utilization in intact cells, a decrease in the total ATP production would have reduced the energetic state of C6 cells, unless ATP utilization was also decreased. Since the cellular ATP content and ATP/ADP ratio remained constant during growth, a simultaneous decrease in the ATP production and consumption fluxes occurs throughout the culture period. Such a concomitant decrease in ATP production and demand without affecting the cellular energetic state has already been described during moderate hypoxia of hepatocytes and cardiomyocytes (Budinger *et al.*, 1996; Chandel *et al.*, 1997). An opposite situation was reported during heart contraction where the rise in ATP utilization went with an increase in oxygen uptake with no detectable change in the energetic state of cardiac muscle. It should be noted that the interpretation of these results, like ours, relied on the measurements of the cellular ATP, ADP, and P_i levels. Finally, the regulation of this phenomenon was attributed to calcium, an activator of both the myosin ATPase and the mitochondrial dehydrogenases, leading to the idea that the cellular respiration is controlled by both the ATP utilization and the delivery of reducing equivalents (for review See Balaban, 1990). In spite of the difficulty to reliably determine the C6 energetic state (see also Erecinska *et al.*, 1993), a similar situation might occur during the growth of C6 cells. Intriguingly, the decrease in the metabolic activities of C6 cells was observed at a early stage of culture (i.e., early exponential growth phase), before confluence was reached. Since the global decrease in ATP production fluxes occurred while cell proliferation was not significantly affected, we suggest that nonproliferation-associated ATP-consuming processes were mainly suppressed.

Glutamine Deprivation and Energetic Metabolism

The transient deprivation of glutamine from the culture medium (up to 7 days of deprivation) induced a drastic inhibition of C6 cell growth, as already demonstrated by Portais *et al.* (1996). At the same time, the lack of glutamine in the culture medium induced a great morphological change of C6 cells. These are considered as bipotential glioblastic cells at early passages, which can evolve into more differentiated cells

expressing either oligodendrocytic or astrocytic characters (Mangoura *et al.*, 1989; Lee *et al.*, 1992). It is now well known that a change in the culture conditions (deprivation of the serum from the culture medium, presence of an inhibitor of microtubule polymerization, different support types) (Antonow *et al.*, 1984), the number of passages (Mangoura *et al.*, 1989), and an advanced confluence stage (Nagamatsu *et al.*, 1996) could all induce a differentiation either in oligodendrocytes or astrocytes. Our results clearly demonstrate that C6 glioma cells cultured in the presence of glutamine express both astrocytic and oligodendrocytic markers. The transient deprivation of glutamine from the culture medium induced a differentiation through the oligodendrocytic pathway. Indeed, correlation between the growth rate, the glucose metabolism, and the differentiation phenotype of C6 cells have been recently described. For instance, C6 subclones, grown in the same culture medium, differentiated either with regard to astrocytes or oligodendrocytes and were characterized as slowly or rapidly growing cell lines, respectively (Nagamatsu *et al.*, 1996). However, our data demonstrate that the depletion-induced differentiation of C6 cells to oligodendrocytes is associated with a marked decrease in the growth rate. This points to a direct role of glutamine limitation on growth arrest, which, in turn, might lead to the modified phenotype.

The question is therefore raised as to the role of this amino acid in the growth arrest of C6 cells. A characteristic of C6 glioma cells from early passages, such as those used in our study, is the lack of the glutamine synthetase, a typical glial enzyme (Parker *et al.*, 1980). Indeed, C6 cells did not synthesize detectable amounts of ^{13}C-glutamine in a glutamine-free medium containing [1-^{13}C]glucose (Portais *et al.*, 1993a), as previously reported by direct enzymatic measurements (Frame *et al.*, 1984). Furthermore, these cells have a very low pyruvate carboxylase activity (Portais *et al.*, 1993b) and therefore cannot efficiently provide carbons to the various anabolic processes required for cell proliferation in the absence of glutamine. Besides the role of glutamine as carbon donor, the nitrogen amide of glutamine is utilized in the synthesis of purine and pyrimidine bases and amino sugars necessary for cell growth. Kovacevic *et al.* (1987) demonstrated the role of glutamine, via aspartate, as a carbon and a nitrogen donor for adenylate synthesis. In Ehrlich cells, which have a low glutaminolytic activity and produce aspartate from glutamine, the role of glutamine for the regeneration of adenylate pools after anoxic periods has been demonstrated (Kovacevic *et al.*, 1988). A similar role of glutamine in C6 cells may be hypothesized, given by the fact that these cells are able to rapidly regenerate their ATP store after an ischemic period (Pianet *et al.*, 1991).

The aim of this work was to better characterize the role of glutamine in the energetic metabolism of C6 glioma cells. Our work demonstrates that glutamine deprivation from the culture medium during 2 to 5 days has no influence on the glycolytic activity of C6 cells. Indeed, at a given cell density, the glucose consumption and lactate production rates were the same for C6 cells cultured both in the presence and absence of glutamine. These results differ from those of Portais *et al.* (1996), in which higher glucose consumption and lactate production were observed in the presence of glutamine than without. Nevertheless, their experimental conditions were different from ours, because glutamine removal was undertaken at cell confluence and was restricted to several hours (Portais *et al.*, 1996). Interestingly, the basal respiratory activity of the cells was lower in the absence (particularly for early culture times) than in the presence of glutamine. Moreover, deprivation of glutamine from the culture medium for 1 to 2 days greatly depressed the total cellular ATP-production flux, without significantly affecting the relative contribution of the oxidative phosphorylation and of the glycolysis to the total ATP synthesis (Fig. 6). This low ATP turnover rate was maintained for longer glutamine deprivation (up to 7 days) (Fig. 6). These results indicate that the energy demand of C6 cells is reduced in the absence of glutamine because of the fact that their growth slows down drastically. The lowering of the respiratory activity of C6 cells in the absence of glutamine was related to an increase in the ATP/ADP ratio. Taken together, our results are in agreement with the classical control of oxidative phosphorylation by the ATP utilization (Brand and Murphy, 1987; Balaban, 1990; Brown, 1992), i.e., in the absence of glutamine, C6 cell growth stayed at a low level, the ATP demand dropped, the ATP/ADP ratio rose, and the respiratory chain activity decreased. Nevertheless, it should be noted that, for deprivation periods longer than 5 days and up to 7 days (i.e., from 25 to 50 $\times$ 10^6 cells/g of beads), the ATP/ADP ratio progressively returned to corresponding values measured for cells grown in the presence of glutamine. Moreover, when glutamine was deprived from the culture medium, the respiratory capacity of the cells was the same as in the presence of glutamine, regardless of the transient deprivation period (i.e., from 10 to 50 $\times$ 10^6 viable cells/g of beads). Therefore,

glutamine is not an essential respiratory substrate for C6 cells under our short-term subculture conditions. This result is in agreement with the very low capacity of C6 glioma cells to convert glutamine into pyruvate and, therefore, the low glutaminolytic activity (Portais *et al.* 1996). The situation in C6 glioma cells appears different from what has been observed for many tumor cells not originating from the brain, such as hepatomas or HeLa cells, in which glutamine is an essential respiratory substrate (Kovacevic and Morris, 1972; Reitzer *et al.*, 1979; McKeehan, 1982). Moreover, the fact that the ATP/ADP ratio increased when glutamine was removed from the culture medium for a short period further emphasizes that glutamine is not an essential respiratory substrate for C6 cells. The withdrawal of an essential substrate from the medium would have reduced the cellular ATP content and decreased the ATP/ADP ratio, as observed for inosine and glutamine in Ehrlich ascites carcinoma cells (Kovacevic *et al.*, 1987).

In conclusion, the present study demonstrates that (1) the mitochondrial oxidative phosphorylation is the major ATP-producing pathway in C6 cells, regardless of the growth phase (e.g., exponential phase versus confluence) and of the differentiation state (e.g., oligodendrocyte differentiation induced by transient glutamine deprivation) and (2) within short subculture period, glutamine does not appear an essential respiratory substrate for C6 cells. Moreover, the respiratory activity of C6 cells is primarily controlled by the cellular ATP utilization. In addition to this classical control of cellular oxidative phosphorylation, we showed that the respiratory capacity of C6 cells is subjected to modulation throughout the short subculture period, regardless of the presence or not of glutamine in the incubation medium. Such a proliferation-related decrease in C6 respiratory capacity may represent an adaptation of the mitochondrial capacity for ATP synthesis to a lowering cellular ATP demand.

ACKNOWLEDGMENTS

The authors wish to thank Nicole Rousse for her excellent technical assistance and Dr. Ray Cooke for his contribution to the editing of the manuscript. M. M. was a recipient of a grant from the Association pour la Recherche contre le Cancer.

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