The inhibitory effect of glutamate on the growth of a murine hybridoma is caused by competitive inhibition of the x_C^- transport system required for cystine utilization

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Abstract

Glutamic acid was found to be growth inhibitory to a murine lymphocyte hybridoma in a concentration-dependent manner from 3 to 12 mM glutamate. At 12 mM glutamate there was a 70% decrease in the specific growth rate of the cells. Attempts to alleviate inhibition or adapt cells to growth in glutamate-based media were unsuccessful. It is proposed that elevated glutamate levels impair adequate uptake of cystine, a critical amino acid for the synthesis of glutathione. Glutathione is required by cells to prevent intracellular oxidative stress. The measured rate of uptake of U- 14 C L-cystine into the cells was found to have the following parameters: $K_m = 0.87$ mM, $V_{max} = 0.9$ nmole/mg cell protein per min. The uptake was sodium independent and resembled the previously described x_C^- transport system, with elevated glutamate levels causing extensive inhibition. Glutamate at a concentration of 1.4 mM caused a 50% decrease in cystine uptake from the serum-free growth medium. Glutamate was taken up from the external medium ($K_m = 20$ mM and $V_{max} = 12.5$ nmole/mg cell protein per min) by the same transport system in a stereo specific, sodium independent manner. Of the amino acids examined, it was found that cystine and homocysteic acid were the most extensive inhibitors of glutamate uptake and that inhibition was competitive. Metabolic profiles of the cells grown in cultures containing enhanced glutamate levels revealed an overall increase in net production of alanine, serine, asparagine and aspartate. A substantially increased specific consumption of glutamate was accompanied by a decreased consumption of cystine, valine and phenylalanine.

The combined kinetic and metabolic results indicate that glutamate and cystine are taken up by the anionic transport system x_C^- . The increasing levels of glutamate in the medium result in a decreased transport of cystine by this system due to competitive inhibition by glutamate.

Introduction

Mammalian cell processes are now used routinely for the production of high-value products such as monoclonal antibodies and recombinant proteins. The value of such processes is related to the attainment of high cell densities as this normally translates to high product yields. In order for these objectives to be met a culture process should minimize the effects of nutrient deprivation and metabolic by-product accumulation (Butler and Jenkins, 1989).

One of the metabolic by-products that leads to growth inhibition in mammalian cell cultures is ammonia. The accumulation of ammonia has been shown to be inhibitory to cell growth and has also been implicated in reduced product formation (Butler et al., 1983; Butler and Spier, 1984; Glacken, 1988). The ammonia arises from the relatively high content of glutamine in most commercial cell culture media (2–6 mM). Glutamine is required as an essential precursor of protein and nucleotide synthesis as well as a substrate for energy metabolism (Zielke et al., 1984; Wice et al., 1981). However, glutamine is chemically

unstable and spontaneously degrades to pyrrolidone carboxylic acid and ammonia (0.2 to 0.6 mM day⁻¹) at 37 °C (Bray et al., 1949; Tritsch and Moore, 1962; Butler and Chistie, 1994). The catabolic utilization of glutamine results in one to two moles of ammonia. Significant degradation of glutamine also occurs at 4 °C, which limits shelf life of the media and makes sterilization by autoclaving impossible (Tritsch and Moore, 1962).

Strategies for growing mammalian cells in low or non-ammoniogenic cultures have included controlled glutamine feeding. Cells in cultures selectively fed with glutamine to maintain a constant low level concentration of the substrate (< 0.5 mM) in the medium have an efficient metabolism with a relatively low ammonia/glutamine metabolic coefficient (Ljunggren and Haggstrom, 1990).

An alternative approach to circumvent the problem of ammonia accumulation is to substitute glutamine by other potential energy substrates. Glutamine metabolism involves deamidation to glutamate prior to deamination or transamination before forming the TCA cycle intermediate, 2-oxoglutarate. Therefore, glutamate is an obvious substitute energy substrate because its carbon/nitrogen ratio is closer to the overall value found in cells. The use of glutamate as a substitute for glutamine has the potential benefit of providing a carbon substrate which leads to the production of a lower amount of ammonia but an equivalent energy production.

The pre-requisites for glutamate utilization for cell growth include a sufficiently high rate of transport and an intracellular glutamine synthetase activity sufficient to provide the minimal cellular requirement for glutamine as a synthetic precursor of protein and nucleic acid. The mechanism of adaptation to glutamate-based media has been shown to be associated with an induction of glutamine synthetase and an enhancement of the rate of transport of glutamate which may be an order of magnitude lower than that of glutamine (McDermott and Butler, 1993). This leads to a decrease in ammonia accumulation and an improved cell yield. The period of adaptation required to attain equivalent or higher cell growth can vary between cell lines (Hassell and Butler, 1990) and supplementation of minimal quantities of other amino acids may be desirable (Christie and Butler, 1999). The failure of one cell line (MDCK) to adapt to glutamate from glutamine was reported to be related to a low and non-inducible rate of glutamate transport (McDermott and Butler, 1993).

Bebbington et al. (1992) developed a protein expression vector for mammalian cells that incorporates the glutamine synthetase gene. This gene as well as a co-transfected gene of choice can be amplified by methionine sulfoximine which competitively inhibits the glutamine synthetase enzyme. This can act as a selective marker because only with a sufficiently high glutamine synthetase activity can cells survive in glutamine-free media. This strategy has proved successful for the amplification and expression of extraneous genes in a number of producer cell lines which are then grown in the absence of glutamine.

Previous work from our laboratory has characterized extensively the metabolic properties of an Sp2/0-derived hybridoma which secretes an antibody against insulin (CC9C10). We have reported on the metabolic energy metabolism of this cell line with respect to the use of glucose and glutamine (Petch and Butler, 1994) as well as other potential carbohydrate sources (Petch and Butler, 1996). In an attempt to circumvent the use of glutamine we reported the usefulness of glutamine-based dipeptides. These provide a slow release of glutamine by the hydrolysis of the dipeptide in the culture medium (Chistie and Butler, 1994; Butler and Christie, 1994). With the appropriate dipeptide concentrations we were able to attain a reduced ammonia production and increased cell yield. The reason that we developed this strategy for these cells was the unexplained apparent toxicity of the addition of glutamate to these cultures. The present report attempts to explain this apparent cellular toxicity by an inhibitory mechanism of a membrane transport system shared by glutamate and cystine.

Materials and methods

Cell line

The murine hybridoma cell line, CC9C10 was obtained from the American Type Culture Collection (No. HB 123). The cells were derived from the fusion of BALB/mouse spleen cells with Sp2/0-Ag14 myelomas. The CC9C10 hybridoma produces IgG₁ class monoclonal antibodies against insulin (Schroer et al., 1983).

Culture conditions

Cells were routinely cultured in 75 cm² T-flasks (Corning) at 37 °C with a 10% CO₂ overlay. Replicate experimental cultures were conducted in 24-well

plates at a volume of 1.5 ml. The serum-free culture medium (NB-SFM) was composed of DMEM/Ham's F12 (1:1 v/v) supplemented with various ingredients according to a formulation previously described (Barnabé and Butler, 1994). The glutamine and glucose concentrations of this medium were 6 mM and 25 mM. The medium also contained 0.1 mM cysteine and 0.1 mM cystine.

Cell enumeration

Viable cell concentrations were determined by the trypan blue exclusion method (Patterson, 1979). A culture sample was mixed with an equal volume of reagent (0.2% trypan blue in D-PBS) and cells were counted with a Neubauer haemocytometer. Total cell concentrations were determined by a Coulter counter (model ZF.). Each sample of a cell suspension was diluted $\times 100$ with Isoton prior to counting.

Radioactive amino acid uptake assay

Cells were harvested from the mid-exponential growth phase of a culture and re-suspended at a concentration of 10^7 cells/ml in 5 ml of Hepes buffered saline (140 mM NaCl, 0.7 mM K_2HPO_4 , 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$) or Hepes buffered choline in which the NaCl was replaced by choline chloride (140 mM). A radioactive amino acid (0.1 μ Ci $U-^{14}C$ L-glutamate or $U-^{14}C$ L-cystine) was added to each cell suspension which was then incubated for a specified time period at 37 °C in an incubator/shaker (New Brunswick). Each assay was stopped by vacuum filtration through a pre-soaked membrane filter (Supor-450; 0.45 μ M pore size and 25 mm diameter from Gelman). The cells which were harvested on the filter were washed twice with ice cold buffer. The filters were placed in scintillation vials containing 5 ml Eco-Fluor scintillation fluid and counted using a Rack-Beta Counter (LKB-Wallac). The protein content of the cells used in the radioactive assay was 0.4 mg/ 10^6 cells.

Amino acid analysis

Amino acids were determined by HPLC (LKB/Pharmacia) using a reverse phase C-18 column (Alltech). The amino acids were pre-derivatized with o-phthalaldehyde based on a previously documented method (Jones and Gilligan, 1983). The derivatives were injected by an autoinjector (Shimadzu SIL-9A) and detected by a fluorescence detector

Figure 1. The effect of glutamate on cell growth. CC9C10 hybridomas were inoculated at 10^5 cells/ml into 1.5 ml serum-free medium contained in 24-well plates. Growth was maintained for 96 h. Cultures contained 6 mM glutamine and varying amounts of glutamate as follows: no glutamate/control (●), 1 mM (□), 3 mM (△), 6 mM (▽), 9 mM (◇) and 12 mM (○). Cell concentrations were determined every 24 h with a Coulter counter. Values are means of triplicate cultures ($\pm$ SEM).

(LKB/Pharmacia). Peaks were analysed by computer using EZ-chrome software (Shimadzu) and compared to an internal standard (L- α -amino-n-butyric acid).

Analysis of cysteine and cystine

Cyst(e)ine was carboxymethylated prior to derivatization with o-phthalaldehyde as described above. The protocol for carboxymethylation was adapted from Birwe and Hesse (1991). Each sample or standard (150 μ l) containing cyst(e)ine was incubated with 10 μ l dithiothreitol (96 mM) for 10 min at room temperature to ensure reduction to cysteine. Iodoacetic acid (10 μ l; 680 mM) was then added and incubated for a further 15 min at room temperature.

Results

The growth inhibition of glutamate and amino adipate

The effect of glutamate on cell growth was determined

Figure 2. The growth inhibition of L-glutamate and L-amino adipate. Cultures were established with varying concentrations of glutamate (●) or amino adipate (■) as described for Figure 1. Each point is a mean value ($\pm$ SEM) of the relative cell yield at 96 h compared to controls for glutamate-supplemented cultures ($n = 3$) and amino adipate-supplemented cultures ($n = 2$).

in a series of cultures in which the CC9C10 hybridomas were inoculated into growth medium which contained 6 mM glutamine and varying concentrations of glutamate up to 12 mM (Figure 1). The cell inoculum was prepared from a culture at exponential phase. This accounted for the absence of any substantial lag phase in the growth curve profiles shown in Figure 1. Cell densities were determined by Coulter counter from samples taken every 24 h over a 96 h culture period.

The inset in Figure 1 shows the decrease in the specific growth rate (μ) at high glutamate concentrations as calculated over the entire culture period. Cultures supplemented with 1 mM glutamate had a similar growth rate to that of the control (0.024 h^{-1}). However, at glutamate concentrations above 1 mM the specific growth rate decreased in proportion to the glutamate added. It would appear that the greatest difference in growth between these cultures occurred within the first 48 h after which the difference decreased. However, all cell counts beyond 96 h declined.

Figure 2 shows the inverse relationship between the maximum cell density and glutamate concentration over the inhibitory range (1–23 mM). This data indicated that the concentration of glutamate resulting in

Figure 3. The rate of uptake of cystine into cells. A radioactive assay was established by suspending CC9C10 cells at $10^7/\text{ml}$ in Hepes buffered saline (○) or Hepes buffered choline (●) containing 0.2 mM L-cystine + $0.1 \mu\text{Ci U-}^{14}\text{C}$ L-cystine. Each assay mixture was incubated at 37°C for a fixed period of time up to 10 min. Cells were collected on a $0.45 \mu\text{M}$ filter and counted for radioactivity by a scintillation counter. Each point is a mean determination $\pm$ SEM ($n = 5$).

half-maximal cell density (IC_{50}) was $10.6 \pm 0.8 \text{ mM}$. It was also shown that L-amino adipate which is an analogue of glutamate inhibited cell growth with an IC_{50} of $4.4 \pm 0.4 \text{ mM}$.

Various attempts were made to adapt the CC9C10 cells to glutamate-based media using alternative strategies. These included growth in media in which glutamine was replaced by glutamate or growth in media containing a minimal level of glutamine. Cells were also passaged into glutamate-based media (containing 0.5 mM gln) at high cell densities ($> 5 \times 10^5/\text{ml}$). However, from these experiments there was no evidence of cellular adaptation to glutamate or an altered inhibitory effect of glutamate.

Amino acid utilization

The effect of a glutamate supplement on the cellular utilisation or production of amino acids was examined by analysis of the media during the growth phase of cultures (24–96 h). Table I shows the specific consumption or production of each amino acid analysed in cultures supplemented with glutamate up to 12 mM. Of note is the observation that in control cul-

Table 1. The specific amino acid production (+) or consumption (–) rates of CC9C10 cells grown in the presence of glutamate

Amino Acid	Control	+ 6 mM Glu	+ 12 mM Glu
μ (specific growth rate, h^{-1})	0.024	0.021*	0.017*
Alanine	24.1 ± 2.1	52.4 ± 7.7	62.0 ± 4.3
Arginine	-4.9 ± 1.7	-4.0 ± 0.3	-3.9 ± 0.9
Asparagine	1.9 ± 0.5	3.6 ± 0.9	6.4 ± 0.4
Aspartate	-1.2 ± 0.3	2.5 ± 0.1	0.9 ± 0.7
Cystine	-4.6 ± 0.5	-3.1 ± 0.4	-2.3 ± 1.0
Glutamate	0.5 ± 1.4	-22.2 ± 13.4	-56 ± 48
Glutamine	-103 ± 19	-106 ± 18	-106 ± 17
Histidine	-0.3 ± 0.3	-1.5 ± 1.1	0.5 ± 0.3
Isoleucine	-2.6 ± 0.6	-1.9 ± 0.8	-1.2 ± 1.6
Leucine	-3.7 ± 0.7	-3.3 ± 0.7	-2.3 ± 1.7
Lysine	-6.0 ± 1.4	-4.0 ± 0.9	-2.6 ± 2.8
Methionine	-3.1 ± 0.8	-2.2 ± 1.2	-2.3 ± 0.9
Phenylalanine	-1.9 ± 0.4	-0.9 ± 0.5	-0.7 ± 0.7
Serine	-2.8 ± 1.2	3.8 ± 1.2	6.2 ± 1.1
Tyrosine	-4.2 ± 1.6	-5.1 ± 2.1	-6.2 ± 2.9
Threonine	-3.3 ± 1.0	-2.3 ± 1.0	-1.5 ± 1.1
Valine	-3.5 ± 0.7	-2.4 ± 0.7	-0.7 ± 1.8

Media samples were analyzed over the exponential growth period (96 h) for cells in cultures containing glutamate at 0, 3 or 12 mM. Each value represents the mean ($\pm$ SEM) of the specific consumption or production rates (nmol/ 10^6 cells per h) for 3 independent cultures.

* significantly different growth rate from the control ($P < 0.01$)

tures there was a low specific rate of production of glutamate (0.5 nmol/ 10^6 cells per h). On the other hand, in glutamate-supplemented cultures, glutamate was consumed at a specific rate proportional to the supplemented concentration and up to 56 nmol/ 10^6 cells per h in cultures containing 12 mM glutamate.

This substantial increase in the specific consumption rate of glutamate caused changes in the production or consumption rates of a number of other amino acids. The specific production of 4 amino acids (ala, asn, asp and ser) increased significantly with glutamate supplementation. In the case of aspartate and serine, the consumption in control cultures was changed to production in the glutamate-supplemented cultures. Of the other amino acids, phe, val and cys appeared to have a significantly reduced uptake in glutamate-supplemented cultures. Glutamine was consumed under all conditions at a rate not significantly different from the control.

In this analysis cystine was measured by the formation of a carboxymethylated OPA derivative. This represented the total cystine or cysteine present in the medium. The specific rates of consumption indicated in Table I are expressed with reference to a cystine

standard. The reduction in the specific uptake rate of cyst(e)ine was considered to be important because of the essential metabolic requirement of cysteine as a precursor of glutathione synthesis.

The kinetic characteristics of cystine uptake

In order to understand the mechanism of inhibition of cell growth, the transport of cystine into the cell was measured under different conditions by a short-time radioactive assay. Cystine (0.2 mM) in the presence of 0.1 μCi U- ^{14}C L-cystine was added to a suspension of 10^7 cells/ml in the sodium-containing buffer (HBS) or in the sodium-independent buffer (HBC). The incorporation of radioactivity into the cells was followed over a period of 10 min (Figure 3). The data shows a linear uptake over the 10 min incubation with a significantly ($\times 3$) rate in the sodium-independent HBC buffer.

The concentration-dependence of cystine uptake was determined over a concentration range of 0.01 mM to 0.4 mM. The low solubility of cystine did not allow a concentration above this range. The assays were conducted over 3 min and stopped by filtration

Figure 4. The concentration dependence of cystine uptake. Radioactive assays were conducted as described in Figure 3 using HBC as the assay buffer. All assays were supplemented with $0.1 \mu\text{Ci U-}^{14}\text{C}$ L-cystine and varying amounts of cold cystine from 0.01 mM to 0.4 mM. Each assay was incubated at 37°C for 3 min. Each point is a mean ($\pm\text{SEM}$) for $n = 3$. The inset shows a double-reciprocal plot (Lineweaver-Burk plot) of the data.

of the cells which were then counted for radioactivity. Figure 4 shows that the data may be modelled with a hyperbolic curve characteristic of Michaelis-Menten kinetics. The K_m value was 0.87 ± 0.1 mM and the $V_{\max}$ was 0.36 ± 0.03 nmole/ 10^6 cells per min ($= 0.9$ nmole/mg cell protein per min).

The effect of glutamate on cystine uptake was determined in a series of assays in which glutamate up to 20 mM was added to assays containing 0.2 mM cystine and $0.1 \mu\text{Ci U-}^{14}\text{C}$ L-cystine. The assays were performed in HBC and in NB-SFM medium (Figure 5). The concentration of cystine used in this experiment (0.2 mM) corresponds to that normally present in NB-SFM medium. The data showed that in both cases there was significant concentration-dependent inhibition of cystine uptake following the addition of glutamate. The kinetic parameters of inhibition were determined by non-linear curve fitting using the Marquardt-Levenberg algorithm of SigmaPlot. The

Figure 5. The effect of glutamate on the uptake of cystine. Radioactive uptake assays were conducted as described in Figure 3 in Hepes buffered choline (●) or NB-SFM medium (■) containing 0.2 mM L-cystine + $0.1 \mu\text{Ci U-}^{14}\text{C}$ L-cystine. Glutamate was added to assays at a concentration range of 0.01 mM to 20 mM. Each point is a mean ($\pm\text{SEM}$) for $n = 3$.

data was fit to an equation:

$$v = V_0 - (V_{\max}[S]/(I_{0.5} + [S]))$$

where v = rate of uptake

where V_0 = rate of uptake without added glutamate

where S = glutamate concentration

where $V_{\max}$ = reduction in rate of uptake of cystine at maximum inhibition

where $I_{0.5}$ = concentration of glutamate causing 50% of the maximum inhibition of uptake.

The experimental data fit this equation with a coefficient of determination (R^2) of 0.999. From the equation the $I_{0.5}$ values in HBC and in NB-SFM were determined to be 2.2 ± 0.8 mM and 1.39 ± 0.07 mM glutamate respectively.

Kinetic characteristics of glutamate uptake

The uptake of glutamate into the cells was studied in a similar radioactive assay as the one used for

Figure 6. The rate of uptake of L-glutamate into cells. A radioactive assay was established by suspending CC9C10 cells at 10^7 /ml in Hepes buffered saline (●) or Hepes buffered choline (○) containing $10 \mu\text{M}$ L-glutamate + $0.1 \mu\text{Ci}$ $\text{U-}^{14}\text{C}$ L-glutamate. Each assay mixture was incubated at 37°C for a fixed period of time up to 10 min. Cells were collected on a $0.45 \mu\text{M}$ filter and counted for radioactivity by a scintillation counter. Each point is a mean determination $\pm\text{SEM}$ ($n = 10$).

cystine uptake. The assays were performed by incubating cells in a buffered solution for short time periods in the presence of $\text{U-}^{14}\text{C}$ L-glutamate. Each assay was terminated by filtering the cell suspension through a $0.45 \mu\text{M}$ filter. After washing, the radioactivity of the filter was measured by scintillation counting. The assays were conducted in the sodium-containing buffer (HBS) and in the sodium-free choline-containing buffer (HBC). The linearity of uptake of L-glutamate was shown both in HBS and HBC over a 10 min time period (Figure 6). A higher rate of uptake of glutamate occurred in HBC and this indicated a sodium-independent transport process.

As a result of this, HBC was selected as the assay medium to study the glutamate concentration dependence of uptake (Figure 7). All the assays were terminated after 10 min and a concentration range of 0.01 mM to 20 mM glutamate was chosen. The kinetics of uptake followed a typical Michaelis-Menten hyperbolic curve. The inset of Figure 7 shows that a double-reciprocal plot results in a straight line as pre-

Figure 7. The concentration dependence of glutamate uptake. Radioactive uptake assays were conducted as described in Figure 6 using HBC as the assay buffer. All assays were supplemented with $0.1 \mu\text{Ci}$ $\text{U-}^{14}\text{C}$ L-glutamate and varying amounts of cold glutamate from 0.01 mM to 20 mM. The assays were terminated after 10 min. Each point is a mean ($\pm\text{SEM}$) for $n = 13$. The inset shows a double-reciprocal plot (Lineweaver-Burk plot) of the data.

dicted from the Lineweaver-Burk transformation. The kinetic parameters of uptake were determined by non-linear curve fitting using the Marquardt-Levenberg algorithm available in SigmaPlot. The K_m and $V_{\max}$ values for this control assay in HBC were determined as 20 mM and 5 nmole/ 10^6 cells per min (= 12.5 nmole/mg cell protein per min) respectively (Table 2).

The effect of other amino acids on the uptake of L-glutamate

The individual effects of various amino acids on the uptake of L-glutamate was determined by the radioactive assay using the sodium-free buffer, HBC. Each amino acid was added at 1 mM to the assay which contained $10 \mu\text{M}$ L-glutamate with $10 \mu\text{Ci}$ $\text{U-}^{14}\text{C}$ L-glutamate. The normalised rates of uptake after a 10 min incubation are recorded in Table 3.

The data shows that of the 20 amino acids tested, significant inhibition of glutamate uptake was shown

Table 2. The kinetic parameters determined for the uptake of glutamate in the presence of potential inhibitors

Conditions	K _m (mM)	V _{max} (nmoles/mg cell protein per min)	K _i (mM)
Glutamate (control)	20 ± 6	13 ± 3	n.d.
Glutamate + cys	36 ± 8	15 ± 3	0.4 ± 0.1
Glutamate + HCA	93 ± 2	15 ± 1	0.14 ± 0.03
Glutamate in NB-SFM	98 ± 5	13 ± 3	n.d.

The parameters were calculated from the data shown in Figure 9. The assays were conducted in Hepes buffered choline (HBC) except for one conducted in the serum-free growth medium (NB-SFM) as indicated. The inhibitors were cystine (cys at 0.2 mM) or homocysteic acid (HCA at 0.4 mM). n.d. = not determined

Table 3. The inhibitory effect of individual amino acids on glutamate uptake

Supplemented amino acid	Uptake rate of L-glu (% of control)
control (no supplement)	100 ± 17
L-alanine	92 ± 15
L-arginine	75 ± 2
L-asparagine	56 ± 5*
L-aspartate	73 ± 10
L-cysteine	66 ± 13
D-glutamate	84 ± 7
L-glutamine	119 ± 18
L-histidine	64 ± 8
L-homocysteic acid	24 ± 4**
L-isoleucine	66 ± 12
L-leucine	82 ± 14
L-lysine	109 ± 14
L-methionine	83 ± 16
L-phenylalanine	112 ± 25
L-proline	62 ± 8
L-pyroglutamate	104 ± 10
L-serine	103 ± 15
L-threonine	64 ± 8
L-tryptophan	97 ± 16
L-valine	65 ± 4

The uptake of 10 μ M L-glutamate (+ 0.1 μ Ci U-¹⁴C-glutamate) into the CC9C10 cells was determined in Hepes buffered choline in the presence of individual amino acids (1 mM). Values indicated are the rates of uptake of glutamate in each assay compared to the control (designated 100). The control was an assay conducted without a supplement where the measured rate was 0.0034 ± 0.0006 nmole/10⁶ cells per min. Each value is a mean ± SEM for n = 3

* significantly different uptake rate from the control at P < 0.1 or ** at P < 0.05

for asparagine (54%; P < 0.1) and homocysteic acid (24%; P < 0.05). Although some other amino acids showed moderate inhibition of glutamate uptake the values were not significantly different from the control as determined by t-test at P < 0.1. The amino acids tested included L-cysteine and two analogues of glutamate (D-glutamate and pyroglutamate). The lack of inhibition of the glutamate analogues showed that the mechanism of uptake of L-glutamate is stereospecific.

Cystine was not used in this experiment because of its low solubility. In a separate experiment cystine was added at 0.2 mM to a range of glutamate concentrations (0.01 mM to 20 mM) in the radioactive uptake assay (Figure 8). This level of cystine was equivalent to that present in NB-SFM. Analysis of the kinetic data showed that the effect of cystine was to competitively inhibit the uptake of glutamate resulting in an increased apparent K_m of 36 mM but an equivalent V_{max} of 6 nmole/10⁶ cells per min (= 15 nmole/mg cell protein per min) (Table 2).

The kinetic uptake experiments were repeated in the presence of homocysteic acid (0.4 mM) and in NB-SFM (Figure 9). Figure 9 shows the Lineweaver-Burk double-reciprocal transformation of this data which clearly shows the typical characteristic of competitive inhibition following the addition of cystine, homocysteic acid or NB-SFM. The inset shows the data at the higher glutamate concentrations. Comparison with the control data showed that the V_{max} was unaffected but the apparent K_m value was increased. Thus, both cystine and homocysteic acid act as competitive inhibitors of glutamate uptake into the cell with K_i values of 4 mM and 9 mM respectively. The data also indicated the presence of competitive inhibitors in the NB-SFM medium. One of these would be cystine but the degree of inhibition suggests the presence of other inhibitors

Figure 8. The effect of cystine on glutamate uptake. Radioactive uptake assays were conducted as described in Figure 6 and using the glutamate concentration range of 0.01 mM to 20 mM described in Figure 7. L-cystine (0.2 mM) was added to each assay. Each point is a mean ($\pm$ SEM) for $n = 12$. The inset shows a double-reciprocal plot (Lineweaver-Burk plot) of the data.

in the medium to produce an apparent K_m value of 98 mM. Although this is similar to the inhibition of homocysteic acid, this amino acid is not present in NB-SFM.

Discussion

In this paper we demonstrate that glutamic acid at a concentration > 1 mM is inhibitory to the growth of a murine hybridoma cell line. This is unusual because this and even higher concentrations of glutamate stimulate the growth of other cell lines such as BHK (Christie and Butler, 1999), McCoy (McDermott and Butler, 1993) and Vero (Hassell and Butler, 1990). The unusual growth properties of hybridoma and myeloma cells have also been recognised by others in the development of glutamine-free media (Bebbington et al., 1992).

We propose that the mechanism of growth inhibition by glutamate in the CC9C10 hybridoma cells is

Figure 9. The inhibitory effects of cystine, homocysteic acid and NB-SFM on glutamate uptake. Lineweaver-Burk double reciprocal plots of kinetic data of U- 14 C L-glutamate uptake are shown for control assays ($\bullet$) and those conducted in the presence of 0.2 mM cystine ($\blacksquare$), 0.4 mM homocysteic acid ($\blacktriangle$) or in NB-SFM ($\blacktriangledown$). The assays were conducted as described in Figure 6. The kinetic data for U- 14 C L-cystine uptake is also shown ($\circ$). This was based on the assay described in Figure 3. The points are means ($\pm$ SEM) of replicate points at each glutamate concentration control ($n = 13$), + cystine ($n = 12$), + homocysteic acid ($n = 2$) and with NB-SFM ($n = 6$).

due to the inhibition of cystine uptake. Cystine has been found to be an essential amino acid for cell lines grown in culture. Exposure of human fibroblasts or mouse leukemia cells to cystine-free media results in rapid death (Bannai, 1986; Bannai et al., 1977). A major metabolic role of cystine is as a precursor for glutathione (γ -glutamyl cysteinylglycine, GSH; Bannai et al., 1977). It has been shown that the intracellular level of glutathione is regulated by the activity of the cystine transport system (Bannai and Tateishi, 1986). Glutathione is a biologically important tripeptide which has been implicated in cellular defense against oxidative damage (Bannai et al., 1977) and as a means of establishing a non-toxic pool of cysteine within the cell (Meredith and Williams, 1986). A low intracellular glutathione content could cause oxidative stress which could lead to a lower growth rate or the induction of apoptosis (Ratan et al., 1994).

Cyst(e)ine can exist in two forms in aqueous solution. Cysteine is the reduced form which contains a sulfhydryl group. Cystine is the oxidised form which contains a disulfide bridge. Cystine is the least soluble amino acid with a concentration of 0.467 mM in a saturated solution at 25 °C (Sano, 1926). Cysteine is freely soluble in aqueous solution but it readily oxidises to cystine in non-reducing solutions (Bannai, 1984). Although cysteine is added to many commercially available culture media, its stable half-life is determined to be no greater than 20–30 min at 37 °C (Fedorcsak et al., 1977). The auto-oxidation of cysteine may be mediated by many factors including low quantities of copper resulting in the formation of cystine.

There are several pieces of evidence that lead us to the conclusion that glutamate causes an inhibition of cystine uptake in the hybridoma cells. The profile of amino acid consumption and production is changed significantly in the hybridoma cultures supplemented with glutamate. Of significance is the decrease in cystine uptake and the switch from glutamate production to glutamate consumption. Over the entire growth period the measured cystine uptake was reduced by 50% in 12 mM glutamate. Although the uptake rates of other amino acids (phe and val) were also reduced, these amino acids are not known to share an uptake mechanism with glutamate and did not appear to cause significant inhibition of glutamate uptake in subsequent experiments.

The sensitivity of the cystine uptake mechanism to glutamate was demonstrated to a greater extent in short-term radioactive uptake experiments. Normal Michaelis-Menten type kinetics were shown for cystine uptake with a K_m of 0.87 mM. The 50% inhibitory concentration of glutamate for the uptake of cystine from culture medium in the uptake assays was determined to be 1.4 mM. The glutamate uptake also showed Michaelis-Menten kinetics but a surprisingly low affinity with a K_m of 20 mM. This is attributed to the fact that the cells used in this assay were taken from cultures containing standard growth medium and at mid-exponential phase. Such cells would have a high intracellular glutamate content and no attempt was made to reduce this prior to assay. Although this probably resulted in low measured glutamate uptake rates, the relative values and conclusions with respect to inhibitory effects were unaffected. Of 21 amino acids tested only cystine and homocysteic acid caused significant inhibition of glutamate uptake with measured K_i values of 0.4 mM and 0.14 mM respectively. The significantly altered apparent K_m values but un-

affected V_{max} in both cases were consistent with the characteristics of competitive inhibition. This suggests that glutamate, cystine and homocysteic acid utilise the same transport mechanism. The stereo-specificity of the system was shown by the lack of inhibition by D-glutamate.

Two glutamate transport systems have been described in mammalian cells. The X_{AG}^- uptake system is characterised as ion-coupled transport specific for glutamate and aspartate. This is sodium-dependent and has been described in various cell types including neuronal cells in which the synaptic release of glutamate and subsequent uptake occurs via this transport system (Dall'Asta et al., 1983; Makowske and Christensen, 1982). The second system is designated x_c^- and was first reported in human diploid fibroblasts (Bannai and Kitamura, 1980). This is a sodium-independent anionic transport system specific for glutamate and cystine but not shared by aspartate. The existence of the x_c^- transport system has been reported in various cells including endothelial cells (Cotgreave and Schuppe-Koisten, 1994), hepatocytes (Christensen and Kilberg, 1987), macrophages (Sato et al., 1995) and CHO cells (Ash et al., 1993). The uptake data reported here for the CC9C10 hybridoma have the characteristics of the x_c^- system which is sodium-independent and shows mutual competitive inhibition for the transport of glutamate, cystine and homocysteic acid but not aspartate.

The availability of cystine for the x_c^- transport system is pH-dependent and can be related to the changed molecular structure of cystine at pH values between 6 and 8. Although the zwitterion form is predominant at the lower pH, the proportion of the anionic form of cystine increases with a rise in pH. At pH 8, 90% of the molecular form is anionic (Bannai and Kitamura, 1982; Bannai, 1984; Reynolds et al, 1991). At pH 7.4 which is the normal starting value for cultures, cystine exists at 70% in the zwitterion form and 30% in the anionic form (net negative charge of -1). Glutamate is in the anionic form with a single net negative charge over the entire range of pH 6 to 8. This is the required form of system x_c^- (van Winkle et al., 1992). Unlike glutamate uptake, cystine uptake was found to exhibit a marked dependence on pH with minimal uptake occurring at pH 6 to a 4-fold increase at pH 8 (Bannai and Kitamura, 1982; Forster and Lloyd, 1985).

The metabolic events associated with the uptake of cystine and glutamate are shown in Figure 10. Upon entering the cells, cystine is rapidly reduced to cysteine and acts as a precursor for the formation

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Figure 10. Transport and metabolic pathways followed by glutamate, glutamine and cystine in mammalian cells.

of glutathione (Bannai, 1986). The transport system x_c^- is capable of transporting cystine or glutamate in either direction across the membrane. However, under normal conditions when glutamine is a nutrient, the transport mechanism acts as an antiporter in which the influx of one mole of cystine is associated with the efflux of one mole of glutamate (Bannai, 1986). Thus a major function of glutamine utilization has been ascribed to the formation of an intracellular glutamate concentration which is slowly released to the extracellular environment and promotes the uptake of cystine via the antiporter (Bannai and Ishii, 1988). This requires the intracellular level of glutamate to remain significantly higher than the extracellular level (Bannai, 1986). Clearly this balance is altered if glutamate is supplemented in the cell culture and as was shown in our data, the normal release of glutamate is changed to an accumulation of glutamate from the medium into the hybridoma cells.

The reason for the particular sensitivity of the hybridoma culture to glutamate-based growth inhibition is uncertain but is probably related to the low intrinsic capacity of the cells for cystine uptake. A low rate of cystine uptake has been previously reported for leukocytes (Rosenberg and Downing, 1965), lymphocytes (Ohmori and Yamamoto, 1982; Burger et al., 1982) and lymphomas (Ishii et al., 1981). A similar mechanism of glutamate-induced cytotoxicity has been previously reported for a neuronal cell line in which the inhibition of cystine uptake resulted in lowered intracellular glutathione levels (Murphy et al., 1989).

The stimulation of cystine uptake has been reported into lymphocytes, bone marrow cells and carcinoma cells by various thiol compounds (Ohmori and Yamamoto, 1983a; Ohmori and Yamamoto, 1983b; Bannai, 1984). The likely mechanism of action is through reduction of cystine in the extracellular medium to cysteine which is transported into the cell via an uptake system other than x_c^- (Ohmori and

Yamamoto, 1983b; Bannai, 1984). However, such an approach would not be suitable in high density cell cultures which require an oxidative environment for growth. Micromolar levels of sodium arsenite have also been reported to increase cystine transport due to the stimulation of the synthesis of a protein analogous to the system x_c^- transporter (Lee et al., 1989; Deneke, 1992).

From the work reported here we show that there are at least three pre-requisites to enable growth of any cell line in glutamine-free media containing glutamate (> 1 mM). The first is that a sufficiently high rate of glutamate transport should be induced to satisfy energy metabolism. The second is that the intracellular glutamine synthetase activity should be sufficient to provide the minimal cellular requirement for glutamine as a synthetic precursor. The third is that glutamate should not interfere with the mechanism that allows sufficient cystine transport into the cell.

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References

- Ash JF, Igo Jr RP, Morgan M and Grey A (1993) Selection of Chinese Hamster Ovary cells (CHO-K1) with reduced glutamate and aspartate uptake. *Som Cell Mol Gen* 19: 231–243.
- Bannai S and Kitamura E (1981) Role of proton dissociation in the transport of cystine and glutamate in human diploid fibroblasts in culture. *J Biol Chem* 256: 5770–5772.
- Bannai S and Kitamura E (1980) Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. *J Biol Chem* 255: 2372–2376.
- Bannai S and Tateishi N (1986) Role of membrane transport in the metabolism and function of glutathione in mammals. *J Membrane Biol* 89: 1–8.
- Bannai S (1986) Exchange of cystine and glutamate across plasma membrane of human fibroblasts. *J Biol Chem* 261: 2256–2263.
- Bannai S (1984) Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta* 779: 289–306.
- Bannai S, Tsukeda H and Okumura H (1977) Effect of antioxidants on cultured human diploid fibroblasts exposed to cystine-free medium. *Biochem Biophys Res Commun* 74: 1582–1588.
- Bannai S and Ishii T (1988) A novel function of glutamine in cell culture: Utilization of glutamine for the uptake of cystine in human fibroblasts. *J Cell Physiol* 137: 360–366.
- Bannai S (1984) Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta* 779: 289–306.
- Bannai S and Kitamura E (1982) Adaptive enhancement of cystine and glutamate uptake in human diploid fibroblasts in culture. *Biochim Biophys Acta* 721: 1–10.
- Barnabé N and Butler M (1994) Effect of temperature on nucleotide pools and monoclonal antibody production in a mouse hybridoma. *Biotech Bioeng* 44: 1235–1245.
- Bebbington CR, Renner G, Thomson S, King D, Abrams D and Yarranton GT (1992) High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Bio/Technology* 10: 169–175.
- Birwe H and Hesse A (1991) High-performance liquid chromatographic determination of urinary cysteine and cystine. *Clin Chimica Acta* 199: 33–42.
- Bray HG, James SP, Raffau IM and Thorpe WV (1949) The enzymatic hydrolysis of glutamine and its spontaneous decomposition in buffer solutions. *Biochem J* 44: 625–627.
- Burger M, Hess MW and Cottier H (1982) The role of 2-mercaptoethanol in the stimulation of spleen cell cultures: increased uptake of cystine into the TCA-soluble pool. *Immunol Lett* 4: 193–197.
- Butler M and Christie A (1994). Adaptation of mammalian cells to non-ammoniogenic media. *Cytotechnology* 15: 87–94.
- Butler M, Imamura T, Thomas J and Thilly WG (1983) High yields from microcarrier cultures by medium perfusion. *J Cell Sci* 61: 351–363.
- Butler M and Jenkins H (1989) Nutritional aspects of growth of animal cells in culture. *J Biotechnol* 12: 97–110.
- Butler M and Spier RE (1984) The effects of glutamine utilisation and ammonia production on the growth of BHK cells in microcarrier cultures. *J Biotechnol* 1: 187–196.
- Christensen HN and Kilberg MS (1987) Amino acid transport across the plasma membrane: role of regulation in interorgan flow. In: *Amino acid transport in Animal Cells*. DL Yudilevich and CAR Boyd, eds. Manchester University Press, Manchester UK. pp. 10–46.
- Christie A and Butler M (1994). Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase. *J Biotechnol* 37: 277–290.
- Christie A and Butler M (1999) The adaptation of BHK cells to a non-ammoniogenic glutamate-based culture medium. *Biotech Bioeng* in press.
- Cotgreave LA and Schuppe-Koistinen I (1994) A role for γ -glutamyl transpeptidase in the transport of cystine into human endothelial cells; relationship to intracellular glutathione. *Biochim Biophys Acta* 1222: 375–382.
- Dall'Asta V, Gazzola GC, Gazzola R, Bussolati O, Longo N and Guidotti GG (1983) Pathways of L-glutamic acid transport in cultured human fibroblasts. *J Biol Chem* 258: 6371–6379.
- Deneke SM (1992) Induction of cystine transport in bovine pulmonary artery endothelial cells by sodium arsenite. *Biochim Biophys Acta* 1109: 127–131.
- Fedorcsak I, Harms-Ringdahl M and Ehrenberg L (1977) Prevention of sulfhydryl autooxidation by a polypeptide from red kidney beans, described to be a stimulator of RNA synthesis. *Exp Cell Res* 108: 331–339.
- Forster S and Lloyd JB (1985) pH-profile of cystine and glutamate transport in normal and cystinotic human fibroblasts. *Biochim Biophys Acta* 814: 398–400.
- Glacken MW (1988) Catabolic control of mammalian cell culture. *Bio/Technology* 6: 1041–1049.
- Hassell T and Butler M (1990). Adaptation to non-ammoniogenic medium and selective substrate feeding lead to enhanced yields in animal cell cultures. *J Cell Sci* 96: 501–508.
- Ishii T, Hishinuma I, Bannai S and Sugita Y (1981) Mechanism of

- growth promotion of mouse lymphoma L 1210 cells *in vitro* by feeder layer or 2-mercaptoethanol. *J Cell Physiol* 107: 283–293.
- Jones BN and Gilligan JP (1983) o-Phthaldialdehyde pre-column derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolyzates and physiological fluids. *J Chromatogr* 266: 471–482.
- Lee TC, Wei ML, Chang WJ, Ho IC, Lo JF, Jan KY and Huang H (1989) Elevation of glutathione levels and glutathione S-transferase activity in arsenic-resistant Chinese hamster ovary cells. *In Vitro Cell Dev Biol* 25: 442–448.
- Ljunggren J and Haggstrom L (1990) Glutamine limited fed-batch culture reduces ammonium ion production in animal cells. *Biotechnol Lett* 12: 705–710.
- Makowske M and Christensen HN (1982) Contrasts in transport systems for anionic amino acids in hepatocytes and a hepatoma cell line HTC. *J Biol Chem* 257: 5663–5670.
- McDermott RH and Butler M (1993) Uptake of glutamate, not glutamine synthetase, regulates adaptation of mammalian cells to glutamine-free medium. *J Cell Sci* 104: 51–58.
- Meredith MJ and Williams GM (1986) Intracellular Glutathione cycling by γ -glutamyl transpeptidase in tumorigenic and non-tumorigenic cultured rat liver cells. *J Biol Chem* 261: 4986–4992.
- Murphy TH, Miyamoto M, Sastre A, Schnaar RL and Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 2: 1547–1558.
- Ohmori H and Yamamoto I (1983a) Mechanism of augmentation of the antibody response *in vitro* by 2-mercaptoethanol in murine lymphocytes: II. A major role of the mixed disulfide between 2-mercaptoethanol and cysteine. *Cell Immunol* 79: 173–185.
- Ohmori H and Yamamoto I (1983b) Mechanism of augmentation of the antibody response *in vitro* by 2-mercaptoethanol in murine lymphocytes: III. Serum-bound and oxidized 2-mercaptoethanol are available for the augmentation. *Cell Immunol* 79: 186–196.
- Ohmori H and Yamamoto I (1982) Mechanism of augmentation of the antibody response *in vitro* by 2-mercaptoethanol in murine lymphocytes. I. 2-Mercaptoethanol-induced stimulation of the uptake of cystine, an essential amino acid. *J Exp Med* 155: 1277–1290.
- Patterson MK Jr (1979) Measurement of growth and viability of cells in culture. *Methods Enzymol* 58: 141–152.
- Petch D and Butler M (1994) A profile of energy metabolism in a murine hybridoma: glucose and glutamine utilization. *J Cell Physiol* 161: 71–76.
- Petch D and Butler M (1996). The effect of alternative carbohydrates on the growth and antibody production of a murine hybridoma. *Appl Biochem Biotech* 59: 93–104.
- Ratan RR, Murphy TH and Baraban IM (1994) Macromolecular synthesis inhibitors prevent oxidative stress induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. *J Neurosci* 14: 4385–4392.
- Reynolds RA, Mahoney SG, McNamara PD and Segal S (1991) The influence of pH on cystine and dibasic amino acid transport by rat renal brush border membrane vesicles. *Biochim Biophys Acta* 1074: 56–61.
- Rosenberg LE and Downing S (1965) Transport of neutral and dibasic amino acids by human leukocytes. *J Clin Invest* 44: 1382–1393.
- Sano K (1926) Solubility of amino acids under the influence of different pH. *Biochem Z* 168: 14–33.
- Sato H, Takenaka Y, Fujiwara K, Yamaguchi M, Abe K and Bannai S (1995). Increase in cystine transport activity and glutathione level in mouse peritoneal macrophages exposed to oxidized low density lipoprotein. *Biochem Biophys Res Comm* 215: 154–159.
- Schroer JA, Bender T, Feldman RJ and Kim KJ (1983) Mapping epitopes on the insulin molecule using monoclonal antibodies; *Eur J Immunol* 13: 693–700.
- Tritsch GL and Moore GE (1962) Spontaneous decomposition of glutamine in cell culture media. *Exp Cell Res* 28: 360–364.
- van Winkle LJ, Mann DF, Wasserlauf HG and Patel M (1992) Mediated Na^+ -independent transport of L-glutamate and L-cystine in 1- and 2-cell mouse conceptuses. *Biochim Biophys Acta* 1107: 299–304.
- Wice BM, Reitzer LJ and Kennell D (1981) The continuous growth of vertebrate cells in the absence of sugar. *J Biol Chem* 256: 7812–7819.
- Zielke HR, Zielke CL and Ozand PT (1984) Glutamine: a major energy source for cultured mammalian cells. *Fed Proc* 43: 121–125.