

Induction of Complete and Irreversible Damage to Malignant Glioma Cells by L-2,4 Diaminobutyric Acid

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Abstract. L-2,4 Diaminobutyric acid (DAB), a non-physiological amino acid, induced an irreversible injury to human malignant glioma cells when incubated for 24h at an amino acid concentration of 6 mmol/l at 37° C. The same treatment of the human glioma cells did not result in any cellular damage; not even a concentration as high as 20 mmol/l of the amino acid under the same incubation conditions did affect these cells. However, a further increase in amino acid concentration above that level resulted in a gradual loss of viability among the human glioma cells. An experimentally induced rat glioma cell line was also affected by DAB, although displaying less sensitivity than the human glioma cells, and complete cellular destruction was achieved at 16 mmol/l of DAB, i.e. well below the critical concentration for human glioma cells. The destructive effect by DAB was pH-sensitive. The N-methylated analogue of a -amino-isobutyric acid exerted a specific inhibitory action on DAB activity, but other amino acids were also inhibitory to various degrees at higher concentrations. Future clinical applications in the treatment of malignant brain tumours in man are discussed.

Neutral, physiological amino acids are transported across the plasma membrane by two or more co-existing transport systems in most cells. The broad-range sodium-dependent System A and the sodium-independent System L are important (1). Essentially, all these amino acids are transported by both System A and System L, although in different proportions. The presence of these two transport systems, therefore, leads to steady states with regard to amino acid transport; so that the net uptake for the amino acids concerned is accomplished by System A and the net exodus by System L (2). Amino acids with weak reactivity with System A and strong affinity for System L will accordingly accumulate only slowly. If the conditions are reversed, however, the result is rather

high cellular levels relative to the extracellular fluid.

The reactivity with the transport systems is dependent on the amino acid structure. The preferred substrates for System A are monoamino acids with short carbon chains; but diamino acids, such as L-2,4 diaminobutyric acid (DAB), can also share this transport system, as has been shown for Ehrlich cells (3). The accumulation pattern in Ehrlich cells by diamino acids being transported against an unusually steep concentration gradient was noteworthy (4). The kinetics of the uptake of DAB in Ehrlich cells were of the nonsaturated type and resulted in cellular damage (5). This basic work by Christensen *et al* was extended and found valid as well for mouse fibrosarcoma cells which were totally and irreversibly damaged by DAB (10 mmol/l) when incubated for 24h at 37° C (6).

The aim of the present work was to investigate the possible effects by DAB on glioma cells under different conditions and on its normal counterparts, the glioma cells. This was relevant in regard to the existing difference in transport capacity mediated by System A between human glioma and glioma cells (7). Therefore, due to the high transporting capacity of neoplastic cells compared to normal cells, another objective was to ascertain conditions under which the DAB concentration was suicidal to the glioma cells while at the same time being harmless for glioma cells.

Material and Methods

Chemicals. All chemicals were of analytical grade. DAB (dihydrochloride form) was from Serva Fine Biochemicals, Federal Republic of Germany. It was dissolved in water and maintained as an isotonic solution (0.1 mmol/l) after pH adjustment to 7.4, with aqueous sodium hydroxide. All other amino acids were obtained from Sigma Chemical Company, USA. **Cell culture.** The growth properties of the human glioma cell line U-178 MG have been described elsewhere (8). The rat glioma line BTSC was established from fetal rat brain tissue after *in vivo* exposure to a single transplacental pulse of the carcinogen N-ethyl-N-nitrosourea (9) (cells were kindly provided by Dr O.D. Laerum, Bergen, Norway). Cells were grown routinely in Eagle's MEM with 10% newborn calf serum and antibiotics (penicillin 100U/ml, streptomycin, 50 µg/ml). Cells were kept at 37° C in humidified air containing 5% CO₂.

For experiments, cells were trypsinized and seeded in serum-containing

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medium into 35 mm plastic petri dishes at a 1:2 split ratio. After 2-3 days of incubation, when confluent monolayers were formed, the medium was changed to serum-free Eagle's MEM (simplified culture medium). After incubation for 24-48 h, cell viability was estimated by light microscopy. Culture media were harvested and cleared by centrifugation for 10 min at 1000 x g and stored at -20° C prior to analysis.

Incubation with DAB and other amino acids. The amino acid tested was added to the glia and glioma cells, when still growing in the simplified, buffered culture medium for incubation in the plates, either alone or together with another amino acid in different concentrations. The simplified culture medium was buffered with 20 mmol/l of HEPES, generally at pH 7.40. Controls were always run concomitantly, i.e., cells maintained for the same time in the simplified, buffered culture medium. Incubation of cells proceeded generally for 24h at 37° C in humidified CO₂/air atmosphere.

The incubation media from control cells (with no additional amino acid) as well as from those with DAB and possibly another amino acid were separated from the cells as given above and saved for lactic acid dehydrogenase (L-lactate: NAD oxido-reductase, E.C. 1.1.1.27, LD) analyses.

Assessment of cell lysis. Cell lysis was assessed by determining LD in culture medium and relating it to the LD found after complete, osmotic lysis (combined with freezing and thawing) of a corresponding amount of cells (6).

Results

Human glioma cells displayed the highest sensitivity to DAB. Complete lysis was obtained at a DAB concentration of 6 mmol/l, with a 50 per cent lysis at around 5 mmol/l after incubation for 24 h at 37° C. The corresponding figures for rat glioma cells were 16 and 12 mmol/l, respectively (Fig. 1).

The higher resistance of rat glioma cells towards DAB in incubation medium was also illustrated by the different slopes of the two curves (Fig. 1). Human glioma cells were not affected at all under these conditions (Fig. 1). Not even at a DAB concentration of 20 mmol/l were any signs of cell necrosis present in the light microscope or in the biochemical LD analysis.

However, when the DAB concentration was increased to 30 mmol/l, an approximate 50 per cent lysis of the glia cells occurred (data not shown in Fig. 1). Similarly, if the incubation period was extended from 24 to 48 h at 37° C, a complete lysis of the human glioma cells occurred at a DAB concentration of 20 mmol/l with a 50 per cent lysis at about 16 mmol/l (data not shown in Fig. 1).

Fig. 2 illustrates the pH profiles for cytolytic activity by DAB in human and rat glioma cells. Human and rat glioma cells were incubated for 24 h at 37° C with 6 and 16 mmol/l of DAB, respectively. Under such conditions the pH curve profiles were practically identical, displaying total inhibition of the cytolytic activity at pH 6.8. Then a sharp increase of activity took place and cytolysis was complete for both types of cells at pH 7.4. This was also true for slightly alkaline conditions up to pH 7.8 (Fig. 2).

2-(Methylamino)-isobutyric acid (MeAIB), containing an N-methyl group, is an exclusive model amino acid for System A (10). Fig. 3 illustrates that this model amino acid had a

distinctly inhibitory effect on the cytolytic activity of DAB on human glioma cells. Most inhibition was already obtained at 2 mmol/l of MeAIB when DAB concentration was 6-8 mmol/l, and 50 per cent inhibition was around 1 mmol/l of MeAIB (Fig. 3). A different curve profile was obtained for human glioma cells (Fig. 3). When these cells were incubated for 24 h at 37° C with 30 mmol/l of DAB a 55 per cent cytolytic activity was achieved. This activity was completely opposed by 10 mmol/l of MeAIB, and the 50 per cent inhibitory activity by the latter amino acid was at a concentration around 3 mmol/l.

The unmethylated 2-amino-isobutyric acid (AIB) is transported mainly by System A in human glia and glioma cells (7). Although being a non-physiological amino acid it was quite harmless to the human glioma cells. Hence, when these cells were incubated for 24 h at 37° C with increasing amounts of AIB up to 10 mmol/l, no signs of cell damage were detected and the LD-values of incubation media were in all instances similar to those of control cells.

AIB, when present in equimolar concentration as DAB in incubation medium, completely inhibited the cytolytic activity of DAB in human glioma cells (Table I). This property of AIB was shared by some other physiological amino acids (Table I). L-leucine, on the contrary, having a very weak interaction with System A (10), had practically no inhibitory action at all opposite to L-norleucine and some other amino acids preferred by System A (Table I).

Discussion

The human glioma cells were totally destroyed by incubation for 24 h with 6 mmol/l of DAB. This was most probably due to a lytic effect on the cells caused by the high osmotic pressure possibly combined with a lowered adenylate charge potential, both phenomena being the result of the energy-dependent, unlimited uptake of DAB. The human glioma cells, on the contrary, did not show any signs of disturbances by the

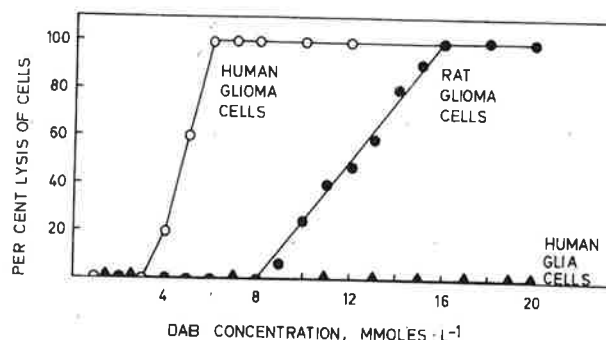


Figure 1. Cytolytic activity of DAB in human (open circles) and rat (filled circles) glioma cells and in human glia cells (filled triangles) after incubation with different concentrations of DAB in simplified culture medium for 24h at 37° C.

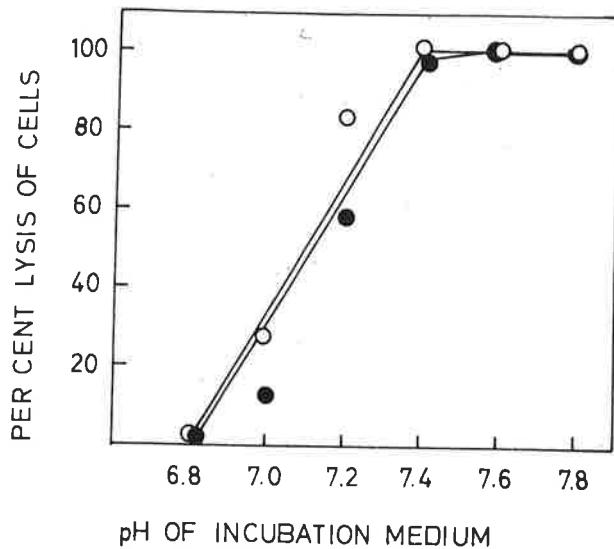


Figure 2. pH dependency of cytolytic activity of DAB on human (open circles) and rat (filled circles) glioma cells. The cells were incubated with 6 (human) and 16 (rat) mmol/l, respectively, of DAB in simplified culture medium for 24h at 37° C.

same treatment as judged in the light microscope and in LD analysis. Not even when the DAB concentration was raised to 20 mmol/l were there any signs of cell necrosis among the glia cells. However, a further increase above the high level in DAB concentration resulted in a gradual decline in viability among these cells. Similarly, a prolongation of the incubation period from 24 h to 48 h also resulted in a complete cellular lysis at a DAB concentration of 20 mmol/l. These data indicate that the observed differences between human glia and glioma cells were in this respect quantitative rather than qualitative. This also tallies with the finding that the increased transport capacity of human glioma cells by System A compared to glia cells was due to an elevated V_{max} -value while the apparent K_m -values were about the same (7). The V_{max} -value corresponds to the amount of operative carriers in the membrane while the apparent K_m -value in this context denotes the affinity of the receptor subunit of the carrier complex to the amino acid (7).

The involvement of System A for DAB transport was further emphasized by the pH-sensitivity of the transport system (3), as well as by the distinctly inhibitory effect by MeAIB; since this latter amino acid is specific for System A (10).

The rat glioma cells did not display the same high sensitivity towards DAB as the human glioma cells; thereby, revealing a certain degree of heterogeneity for the two types of glioma cells. This also implicates that different human glioma cell lines may vary with respect to DAB sensitivity. Such an observation would be in line with many other discordant characteristics found among human glioma cells *in vitro* (11).

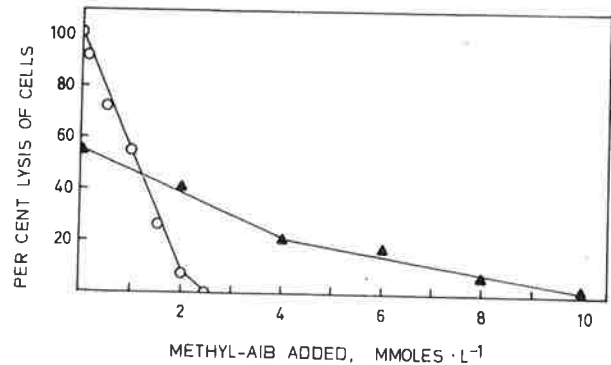


Figure 3. Inhibitory activity on DAB-induced cytotoxicity by different concentrations of N-methyl AIB (Me-AIB). Human glioma (open circles) and glia (filled triangles) cells were incubated with 8 and 30 mmol/l, respectively, of DAB in simplified culture medium containing various amounts of Me-AIB as indicated on abscissa. Incubation for 24h at 37° C.

DAB may prove useful for future treatment of malignant brain tumours in man, since apart from its directed destructive effect on glioma cells *in vitro*, it represents a new principle of action. Thus, the only subcellular organelle primarily involved in its working principle is the plasma membrane. While this synthetic amino acid is in the extracellular fluid, it is harmless to the cells. Other synthetic, non-physiological amino acids, such as Me-AIB or AIB, do not share the property of DAB in being a suicidal substrate to the glioma cells, since they display saturation kinetics (7,10). Our present results also showed that AIB in the incubation medium did not interfere with cell viability among the human glioma cells. Instead AIB and especially Me-AIB together with some other amino acids were all capable of inhibiting the

TABLE I. Effect of additional amino acid in incubation medium on cytolytic activity of DAB. Incubation for 24 h at 37° C of human and rat glioma cells with 8 and 15 mmol/l, respectively, of DAB. Additional amino acid was in equimolar concentration.

Additional amino acid	Human glioma cells, % lysis	Rat glioma cells, % lysis
No additional amino acid	100	100
AIB	0	2
L-Alanine	3	6
L-Methionine	0	5
L-Threonine	3	1
L-Proline	0	0
L-Thioproline	45	52
L-2-Aminobutyric acid	0	0
L-4-Aminobutyric acid (GABA)	28	42
L-Norleucine	4	0
L-Leucine	92	100

lytic effect by DAB on the cells thereby making the DAB administration controllable.

Because of its unique principle of action it might work synergistically against malignant brain tumours with other known therapeutic agents having other subcellular targets for their actions. An appropriate stage for such a future treatment would seem to be directly after the surgical removal of the tumour mass. During this postoperative period the flux of amino acids from the blood plasma compartment into brain is facilitated due to a decreased blood-brain-barrier action. The malignant glioma cells, disseminated in the brain parenchyma, would then be the targets for DAB action.

However, a stumbling block in such a treatment regimen is the aforementioned heterogeneity among the malignant glioma cells themselves (11), here illustrated by the rather sharp differences between human and rat glioma cells in sensitivity towards DAB. A maximal discrepancy in sensitivity between malignant and normal cells is advantageous to achieve a maximum of directed activity against the tumour cells with a minimum of damage to normal cells. Due to experimental difficulties in maintaining cells viable for several days, it was not possible to incubate the human glioma cells for a prolonged period of time (several days) to investigate whether it was possible to "compensate" a sub-optimal concentration of DAB, e.g. 1-2 mmol/l, with length of incubation period.

No curative treatment of malignant gliomas is presently available. Therefore, it is necessary to find new leads on the treatment of this malignant disease. The management by

DAB may offer new possibilities, although many problems remain unsolved.

References

- 1 Oxender DL and Christensen HN: Distinct mediating system for the transport of neutral amino acids by the Ehrlich cell. *J Biol Chem* 238: 3686-3699, 1963.
- 2 Christensen HN: Metabolite transport at cell membranes. In (Levi G, Battistin L, and Lajtha A, eds) *Transport phenomena in the nervous system*, New York, Plenum Press, 1976, pp 3-12.
- 3 Christensen HN: *Biological transport* (2nd edition). WA Benjamin Inc, Massachusetts, 1975.
- 4 Christensen HN, deCespedes C, Handlogten ME, and Ronquist G: Energization of amino acid transport, studied for the Ehrlich ascites tumour cell. *Biochim Biophys Acta* 300: 487-522, 1973.
- 5 Christensen HN: Biochemistry of membrane transport. In: *FEBS symposium No 42* (Semenza G and Carafoli E, eds) Berlin, Heidelberg, New York: Springer, 1977, pp 222-335.
- 6 Ronquist G, Hugosson R, and Westermark B: Antitumour activity of L-2,4 diaminobutyric acid against mouse fibrosarcoma cells *in vitro* and *in vivo*. *J Cancer Res Clin Oncol* 95: 259-268, 1980.
- 7 Ronquist G, Ågren G, Pontén J, and Westermark B: α -Aminoisobutyric acid transport into human glioma and glioma cells in culture. *J Cell Physiol* 89: 433-440, 1976.
- 8 Westermark B: The deficient density - dependent growth control of human malignant glioma cells and virus - transformed glioma-like cells in culture. *Int J Cancer* 12: 438-451, 1973.
- 9 Laerum OD and Rajewsky MF: Neoplastic transformation of fetal rat brain cells in culture after exposure to ethylnitrosourea *in vivo*. *J Natl Cancer Inst* 55: 1177-1187, 1975.
- 10 Christensen HN: Amino acid transport systems in animal cells. Interrelations and energization. *J Supramol Struct* 6: 205-213, 1977.
- 11 Pontén J and Westermark B: Properties of human malignant glioma cells *in vivo*. *Med Biol* 56: 184-193, 1978.

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