Diminished Excretion of Polyamines from BHK-21/C13 Cells Exposed to Methylglyoxal Bis(guanylhyazone)

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Methylglyoxal bis(guanylhyazone) {1,1'-[(methylethanediyldine)dinitrilo]diguanidine} inhibited the growth of BHK-21/C13 cells in monolayer cultures. Accumulation of spermidine and spermine was inhibited, whereas the accumulation of putrescine was increased. The intracellular spermidine/spermine molar ratio decreased only slightly after exposure of the cells to 20 μ M-methylglyoxal bis(guanylhyazone) for 1 day. Cells incubated in the presence of the drug released less polyamine into the culture medium than did control cells, the polyamine released consisting almost exclusively of spermidine, both free and as a conjugated form.

When the growth of BHK-21/C13 cells in monolayer cultures is inhibited by depriving the cells of serum, the intracellular polyamine content falls and the molar ratio of spermidine/spermine decreases. There is concomitant loss of spermidine from the cells into the culture medium (Melvin & Keir, 1977, 1978).

The present study was undertaken to examine the fate of intracellular polyamines when the growth of BHK-21/C13 cells is inhibited by incubating the cells in the presence of methylglyoxal bis(guanylhyazone) {1,1'-[(methylethanediyldine)dinitrilo]diguanidine}, a potent anti-tumour agent (Mihich, 1963). The mechanism of action of this agent on cells is different from that of serum deprivation (Holley & Kiernan, 1974). Methylglyoxal bis(guanylhyazone) is itself a polyamine, resembling spermidine and spermine in structure; it has been shown to inhibit the pathway of biosynthesis of spermidine and spermine (Williams-Ashman & Schenone, 1972) and to act as a competitive inhibitor of spermidine and spermine function (Clark & Fuller, 1975).

Experimental

Materials

[1,4(n)-³H]Putrescine dihydrochloride (19Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive hydrochlorides of putrescine, spermidine and spermine and dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. L-Proline was from BDH Biochemicals, Poole, Dorset, U.K., and methylglyoxal bis(guanylhyazone) dihydrochloride monohydrate was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Tissue-

culture materials, including horse serum screened for mycoplasma, were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and silica-gel 60 t.l.c. plates (20cm×20cm) from Merck, Darmstadt, Germany.

Cell culture

BHK-21/C13 cells (McPherson & Stoker, 1962) were grown in monolayer cultures at 37°C in a 5% CO₂ atmosphere in Eagle's medium, Glasgow modification, containing 10% (v/v) tryptose phosphate broth and 10% (v/v) horse serum [ETH₁₀ medium (Melvin & Keir, 1978)]. Methylglyoxal bis(guanylhyazone) was dissolved in 0.9% NaCl, the pH adjusted to 7.2 and the solution sterilized by filtration, immediately before use.

Analytical determinations

Protein, RNA and DNA were extracted from cells and determined quantitatively as described by Munro & Fleck (1966). Putrescine, spermidine and spermine, extracted from cells by 0.2M-HClO₄, were converted into their dansyl derivatives and separated by t.l.c., the chromatograms being developed twice in ethyl acetate/cyclohexane (2:3, v/v). The amines were determined quantitatively by measurement of the fluorescence of their dansylated derivatives (Herbst & Dion, 1970).

Intracellular polyamines were labelled by incubating non-confluent cells, growing in 2.24-litre roller bottles in ETH₁₀ medium, for 20h, in the presence of [1,4(n)-³H]putrescine dihydrochloride (1.0 μ Ci/ml). The radioactive medium was poured off and the cell sheet washed twice with ETH₁₀ medium before harvesting. Cells were dispensed into 90mm-diameter vented plastic dishes at a density of 0.75×10⁶ cells

per dish and incubated overnight in ETH_{10} medium, after which the medium was replaced with fresh medium. After incubation for the required time, the culture medium was decanted from the cell sheet and centrifuged at $200g_{av}$ for 5 min to remove cell debris. Portions of the supernatant medium were (a) assayed directly for radioactivity, (b) treated with ice-cold 0.2M-HClO_4 and the extracted amines dansylated, separated by t.l.c. and analysed for radioactivity (Melvin & Keir, 1978) or (c) washed with ice-cold trichloroacetic acid (final concn. 4%, w/v) followed by anhydrous ether, and the extracts hydrolysed with 6M-HCl at 110°C for 16 h, then dried to powder form (Gerner & Russell, 1977). Samples were redissolved in 0.2M-HClO_4 , treated with dansyl chloride, and the products separated and analysed for radioactivity. The cell sheet was washed three times with ice-cold phosphate-buffered saline, pH 7.2 (Melvin & Keir, 1978) and the cells were scraped off the dishes. The intracellular amines were extracted either with ice-cold 0.2M-HClO_4 or trichloroacetic acid followed by hydrolysis with HCl ; the amines were dansylated and analysed as described above.

Samples were assayed for radioactivity by liquid-scintillation spectrometry, by using a Triton X-100/toluene (1:2, v/v) scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per litre, in an Inter-technique SL40 liquid-scintillation spectrometer with approx. 40% efficiency for ^3H .

Results

BHK-21/C13 cells, growing in monolayer cultures in 90 mm-diameter plastic dishes, were transferred to fresh medium containing either no drug or $20\mu\text{M}$ -methylglyoxal bis(guanylhydrazone). DNA, RNA, protein and cell number were determined for each culture before transfer (the initial culture value) and 1 day after transfer. There was no evidence of cell lysis, as determined by microscopic examination and impermeability of the cells to Trypan Blue. Cell proliferation and the accumulation of macromolecules were inhibited by methylglyoxal bis(guanylhydrazone) (Table 1).

Putrescine, spermidine and spermine were determined in the cultures described in Table 1. Accumulation of spermidine and spermine was inhibited severely by methylglyoxal bis(guanylhydrazone), whereas the accumulation of putrescine was increased (Table 2). These results suggest that syntheses of spermidine and spermine were inhibited by the drug, whereas synthesis of putrescine continued. In the absence of the drug the intracellular spermidine and spermine contents each increased by approx. 350% within 27 h, whereas in the presence of the drug the spermidine content increased by only 43% and the spermine content by only 56%. The molar ratio of spermidine to spermine decreased slightly in cultures exposed to methylglyoxal bis(guanylhydrazone) compared with control cultures (no drug).

Cells whose polyamines had been labelled with ^3H , by prior incubation with [^3H]putrescine, were transferred to fresh medium containing either no drug or $20\mu\text{M}$ -methylglyoxal bis(guanylhydrazone), and the cells and the medium in which they were incubated were analysed for radioactivity 18 or 24 h later. Cells exposed to the drug lost less radioactivity into the culture medium than did control cells (Table 3).

^3H -labelled BHK-21/C13 cells were incubated for 46 h in the absence or presence of $20\mu\text{M}$ -methylglyoxal bis(guanylhydrazone). Portions of the cells and the medium in which they were incubated were extracted

Table 1. *Effect of methylglyoxal bis(guanylhydrazone) on cell proliferation and accumulation of protein, DNA and RNA in BHK-21/C13 cells*

The results are presented as content per culture dish, four dishes being assayed in triplicate for protein, DNA and RNA. Two dishes were used for each determination of cell number.

	$10^{-6} \times \text{Cell no.}$	Protein (mg)	DNA (μg)	RNA (μg)
Initial culture	1.7	0.33	17.5	50.4
No drug	5.5	1.63	91.6	273.6
$20\mu\text{M}$ -Methylglyoxal bis(guanylhydrazone)	3.8	1.00	48.7	136.8

Table 2. *Effect of methylglyoxal bis(guanylhydrazone) on the accumulation of putrescine, spermidine and spermine in BHK-21/C13 cells*

Four dishes were used for determination of putrescine, spermidine and spermine, the values presented here being the average content per dish from triplicate assays. All estimates varied by less than 10% of the values shown.

	Polyamine (nmol)			
	Putrescine	Spermidine	Spermine	Spermidine/spermine
Initial culture	0.95	6.1	2.7	2.24
No drug	5.25	27.2	12.4	2.19
$20\mu\text{M}$ -Methylglyoxal bis(guanylhydrazone)	7.63	8.7	4.2	2.06

with ice-cold 0.2M-HClO₄, the extracts treated with dansyl chloride, and the products separated by t.l.c. In all samples of cells and medium, much of the radioactivity was found in material which migrated no further than 1.0cm from the origin of the chromatograms (Table 4a). Cells incubated in the presence of methylglyoxal bis(guanylhyazone) had a larger proportion of their radioactivity in this material than cells incubated in the absence of the drug. The remainder of the radioactivity inside the cells was distributed between spermidine and spermine. In control cells, about three times as much radioactivity was present in spermine as in spermidine after 46h, whereas in cells exposed to methylglyoxal bis(guanylhyazone) for 46h spermine and spermidine contained almost equal amounts of radioactivity. This is consistent with the known inhibition by methylglyoxal bis(guanylhyazone) of the synthesis of spermine from spermidine (Williams-Ashman & Schenone, 1972). In the samples of medium, both from control cells and from cells incubated in the presence of the drug, the remainder of the radioactivity was located almost exclusively in spermidine (Table 4a). This indicates that loss of intracellular polyamines was not due to cell lysis, otherwise a large amount of the radioactivity in the medium would have been located in spermine.

Portions of the cells and medium were washed with ice-cold trichloroacetic acid, the extracted material was hydrolysed with 6M-HCl, and the products were dansylated and separated by t.l.c. For all samples, hydrolysis decreased the amount of radioactivity found at the origin of the chromatograms and increased the amount of radioactivity located in spermidine and spermine (Table 4b), which suggests that this material represented conjugated polyamines

(Rosenblum & Russell, 1977). Although the cells now contained more radioactivity in spermine than in spermidine, when incubated in either the absence or presence of methylglyoxal bis(guanylhyazone), the radioactivity in the medium was located predominantly in spermidine (Table 4b). This suggests that spermidine was being excreted specifically from the cells into the culture medium (Melvin & Keir, 1978).

Discussion

When the growth of BHK-21/C13 cells in monolayer cultures was inhibited by methylglyoxal bis(guanylhyazone), the accumulation of spermidine and spermine was prevented, but after 27h there was

Table 3. Effect of methylglyoxal bis(guanylhyazone) on loss of polyamines from BHK-21/C13 cells into the culture medium

The results are presented as radioactivity per dish, each value being the mean of three separate determinations. All values obtained varied by less than 10% of the mean values. All samples, from cells and medium, were assayed for radioactivity using 10ml of Triton X-100/toluene-based scintillation fluid, containing 0.1 ml of 0.2M-HClO₄ and 1.0ml of ETH₁₀ medium, so that the efficiency of counting was the same for all samples.

Drug	Time (h)	Radioactivity in medium	
		(c.p.m.)	(% of total)
None	18	4791	9.1
	24	5848	9.9
20µM-Methylglyoxal bis(guanylhyazone)	18	3234	5.8
	24	3397	5.8

Table 4. Distribution of ³H-labelled material in extracts of BHK-21/C13 cells and their incubation medium after 46h in the presence or absence of 20µM-methylglyoxal bis(guanylhyazone)

The initial radioactivity in the cells at 0h was 47.5 × 10³ c.p.m. per dish. After 46h, control cells (no drug) had lost approx. 15.3 × 10³ c.p.m. into the culture medium per dish, and cells incubated in the presence of 20µM-methylglyoxal bis(guanylhyazone) had lost approx. 5.9 × 10³ c.p.m. per dish.

Methylglyoxal bis(guanylhyazone) (µM)		Radioactivity (%)			
		Origin	Spermine	Spermidine	Putrescine
(a) HClO ₄ extracts					
Cells	0	16	63	20	1
	20	31	36	32	1
Medium	0	50	3	37	10
	20	64	1	27	8
(b) 6M-HCl hydrolysates					
Cells	0	11	66	22	1
	20	10	50	39	1
Medium	0	11	13	63	13
	20	10	5	80	5

little change in the molar ratio of spermidine to spermine in cells exposed to the drug compared with control cells, and the cells lost even less polyamines into the culture medium than did control cells. These results are markedly different from those obtained with BHK-21/C13 cells whose growth rate had been inhibited by deprivation of serum (Melvin & Keir, 1978), which is consistent with the different modes of action of these inhibitors.

The ^3H -labelled material released from BHK-21/C13 cells exposed to methylglyoxal bis(guanylhydrazone) was almost exclusively spermidine, both free and as a conjugated form. Conjugation took place inside the cells, and might be a prerequisite for excretion (Rosenblum & Russell, 1977). Excretion of spermidine from BHK-21/C13 cells is an important mechanism by which the cells dispose of polyamines present in excess of their requirements (Melvin & Keir, 1978). In BHK-21/C13 cells whose growth has been inhibited by methylglyoxal bis(guanylhydrazone) inhibition of biosynthesis of spermidine and spermine and their accumulation might result in insufficient spermidine and spermine being available for continuation of cell proliferation (Heby *et al.*, 1977). To conserve intracellular polyamines, and hence maintain cell growth rate, the mechanism of transport of spermidine out of the cells might be inhibited.

Methylglyoxal bis(guanylhydrazone) is a competitive inhibitor of spermidine and spermine function (Clark & Fuller, 1975) and can inhibit transport systems for the uptake of exogenous spermidine into mammalian cells in culture (Dave & Caballes, 1973; Clark & Fuller, 1975). It may be that in BHK-21/C13

cells whose growth has been inhibited by the drug, intracellular methylglyoxal bis(guanylhydrazone) acts as a competitive inhibitor of the transport of spermidine out of the cells.

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