Polyamines in mycoplasmas and in mycoplasma-infected tumour cells

Leena ALHONEN-HONGISTO,* Pirjo VEIJALAINEN,† Christine EK-KOMMONEN† and Juhani JÄNNE*

*Department of Biochemistry, University of Helsinki, SF-00170 Helsinki 17, and †State Veterinary Medical Institute, SF-00550 Helsinki 55, Finland

(Received 27 August 1981/Accepted 12 October 1981)

Three out of four different mycoplasma strains analysed for the polyamine contents contained relatively high concentrations of putrescine, cadaverine, spermidine and spermine. In addition to ornithine decarboxylase (EC 4.1.1.17) activity, the mycoplasmas also exhibited comparable or higher lysine decarboxylase (EC 4.1.1.18) activity fully resistant to the action of 2-difluoromethylornithine, an irreversible inhibitor of eukaryotic ornithine decarboxylase. 2-Difluoromethylornithine did not modify the polyamine pattern of actively growing mycoplasmas. Ehrlich ascites carcinoma cells and L1210 mouse leukemia cells infected with any of the four mycoplasma strains contained, in addition to putrescine, spermidine and spermine, and also easily measurable concentrations of cadaverine; the latter diamine was absent in uninfected cultures. When the infected cells were exposed to difluoromethylornithine, the accumulation of cadaverine was markedly enhanced. The modification of cellular polyamine pattern by mycoplasmas, especially in the presence of inhibitors of eukaryotic ornithine decarboxylase, could conceivably be used as an indicator of mycoplasma infection in cultured animal cells.

Apart from the fact that, although not essential, spermine (or spermidine) distinctly stimulates growth of certain mycoplasma strains (Rodwell, 1967), virtually nothing is known about the occurrence or metabolism of polyamines in these primitive organisms.

Although the biosynthetic pathway leading to the formation of polyamines is markedly similar in eukaryotic and prokaryotic organisms, differences exist especially with respect to the regulation of ornithine decarboxylase in bacterial and animal cells. Mammalian ornithine decarboxylase activity is thought to be regulated mainly by fluctuations in the amount of active enzyme protein (Jänne *et al.*, 1978), whereas the activity of the bacterial enzyme is profoundly influenced, positively or negatively, by certain nucleotides (Hölttä *et al.*, 1972, 1974).

In the present paper, an analysis of several mycoplasma strains revealed that these organisms contain substantial amounts of the natural polyamines, including cadaverine. The mycoplasmas likewise exhibited both ornithine decarboxylase and lysine decarboxylase activities that were insensitive to nucleotides and largely to 2-difluoromethylornithine. The finding that mycoplasma infection of animal cells results in the appearance of cadaverine,

especially when the cell's own putrescine and spermidine synthesis was inhibited, is a reason for caution in the interpretation of our recent observation indicating that polyamine depletion by 2-difluoromethylornithine in Ehrlich ascites carcinoma cells induced an enhanced accumulation of cadaverine and its aminopropyl derivative (Alhonen-Hongisto & Jänne, 1980).

Experimental

Cell cultures

Mycoplasma suis pneumoniae strain previously described (Friis, 1969) was kindly provided by Dr. N. F. Friis (State Veterinary Serum Laboratory, Copenhagen, Denmark). The bovine mycoplasma cultured from lungs of a healthy calf was a gift from Dr. T. Eskola (State Veterinary Medical Institute, Helsinki). The mycoplasmas of rat were isolated from lungs of two sick laboratory animals.

Since the isolation, the strains have been subcultured several times in liquid medium. *M. suis* pneumoniae was grown in N-medium (Friis, 1971) containing 10% heat-inactivated (56°C for 30 min) newborn-calf serum and 10% horse serum. For the other strains, PPLO broth containing meat extract,

yeast extract, glucose and 20% horse serum was used.

Each culture was tested for viable mycoplasmas as colony-forming activity on solid mycoplasma agar plates. The absence of bacterial and fungal contamination was confirmed by cultivation on blood and Sabouraud agars. In all experiments the mycoplasmas were in actively growing phase. All the experiments were performed at least twice.

For the preparation of cell extracts the mycoplasmas were harvested by centrifugation (15000g for 10 min at 5°C) and suspended in 25 mM-Tris/HCl buffer (pH 7.4), containing 0.1 mM-EDTA and 1 mM-dithiothreitol. The cells were disintegrated ultrasonically and cytosol fractions were prepared as described by Alhonen-Hongisto *et al.* (1979).

Ehrlich ascites carcinoma cells were adapted to growth in suspension cultures in RPMI 1640 medium (Orion Diagnostica, Helsinki) supplemented with 5% pooled human serum (Finnish Red Cross Transfusion Service, Helsinki) and 50 µg of both penicillin and streptomycin/ml (Alhonen-Hongisto *et al.*, 1979). L1210 mouse leukaemia cells, obtained from Flow Laboratories, were likewise cultured in RPMI 1640 medium with the aforementioned supplementation.

Cell cultures were infected with mycoplasmas by adding actively growing mycoplasma suspensions.

The tumour cells were harvested, disintegrated ultrasonically and the extracts for enzyme assays and polyamine measurements were prepared as described by Alhonen-Hongisto *et al.* (1979).

Chemicals

DL-[1-¹⁴C]Ornithine (sp. radioactivity 59 Ci/mol) and L-[U-¹⁴C]lysine (sp. radioactivity 341 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. DL-2-Difluoromethylornithine was a generous gift from the Centre de Recherche Merrell International (Strasbourg, France).

Analytical methods

The activity of ornithine decarboxylase was assayed as described by Jänne & Williams-Ashman (1971). The activity of lysine decarboxylase was measured by using identical incubation conditions, but replacing ornithine by radioactive lysine.

Polyamines were measured after dansylation (Seiler, 1970) employing the solvent system as described by Alhonen-Hongisto & Jänne (1980).

Results

As shown in Table 1, the different mycoplasma strains analysed contained substantial amounts of the polyamines, yet quantitative differences existed. Rodent and bovine mycoplasmas contained, in addition to putrescine, spermidine and spermine, also relatively high concentrations of cadaverine, whereas in *M. suipneumoniae* putrescine appeared to be the only polyamine. An exposure of the mycoplasma cultures to 3 mM-2-difluoromethylornithine for several days did not inhibit, or even enhance, the accumulation of the polyamines.

Rat and bovine mycoplasma exhibited readily measurable activities of ornithine decarboxylase and lysine decarboxylase, the latter being unaffected by 1 mM-2-difluoromethylornithine in the assay mixture (Table 2). Unlike ornithine decarboxylase from *Escherichia coli* (Hölttä *et al.*, 1972), mycoplasma enzyme was not stimulated by nucleotides (results not shown). The view that separate enzymes were responsible for the decarboxylation of ornithine and lysine was supported by the finding indicating that a 50-fold excess of unlabelled lysine did not inhibit the decarboxylation of ornithine and similar excess of ornithine did not inhibit the decarboxylation of lysine by mycoplasma extracts (results not shown).

The inhibition of ornithine decarboxylase by 2-difluoromethylornithine in rat mycoplasma cultures (Table 2) was inconsistent and varied in different cultures. In addition to ornithine and lysine

Table 1. Polyamine concentrations in mycoplasmas grown in the absence or presence of 2-difluoromethylornithine. Mycoplasma cultures were diluted 1:10 with fresh medium, grown for 2 days and then supplemented with 3 mM-2-difluoromethylornithine (DFMO). Polyamines were determined 2 days thereafter. Abbreviation: n.d., not detected.

Strain	Treatment	Content (nmol/mg of DNA)			
		Putrescine	Cadaverine	Spermidine	Spermine
Rat mycoplasma (2A)	—	87	120	1862	27
	DFMO	80	80	1443	28
Rat mycoplasma (682)	—	69	108	1494	54
	DFMO	48	113	1361	48
Bovine mycoplasma	—	78	58	536	10
	DFMO	234	206	694	20
<i>M. suipneumoniae</i>	—	1735	n.d.	n.d.	n.d.
	DFMO	3292	n.d.	n.d.	n.d.

Table 2. *Ornithine decarboxylase and lysine decarboxylase activities in actively growing rat and bovine mycoplasmas*
After dilution with medium, mycoplasma cultures were grown for 24 h, whereafter the cells were harvested and enzymes were assayed in the absence or presence of 1 mM-2-difluoromethylornithine (DFMO).

Strain	Addition	Activity (pmol/mg of protein per h)	
		Ornithine decarboxylase	Lysine decarboxylase
Rat mycoplasma	—	35.9	52.9
	DFMO	18.8	50.9
Bovine mycoplasma	—	254	3860
	DFMO	229	4030

Table 3. *Effect of mycoplasma infection on polyamine concentrations in cultured Ehrlich ascites carcinoma cells (A) and L1210 mouse Leukaemia cells (B) with normal or depleted polyamine pattern*
Cultures in Ehrlich ascites cells (initial density 0.18×10^6 cells/ml) and L1210 cells (initial density 0.13×10^6 cells/ml) were infected with mycoplasmas and grown for 48 h in the absence or presence of 3 mM-2-difluoromethylornithine (DFMO). Abbreviation: n.d., not detected.

Infecting mycoplasma strain	Treatment	Content (nmol/mg of DNA)				
		Putrescine	Cadaverine	Spermidine	Aminopropylcadaverine	Spermine
(A)	—	170	n.d.	340	n.d.	112
	DFMO	9	n.d.	112	n.d.	148
	Rat mycoplasma (2A)	247	55	350	n.d.	98
	DFMO	17	164	85	18	172
	Rat mycoplasma (682)	313	174	317	n.d.	137
	DFMO	181	473	244	n.d.	164
	Bovine mycoplasma	180	47	312	n.d.	89
	DFMO	51	154	267	n.d.	158
	<i>M. suipneumoniae</i>	204	6	259	n.d.	105
	DFMO	32	19	234	n.d.	180
(B)	—	27	n.d.	213	n.d.	70
	DFMO	11	n.d.	25	n.d.	154
	Rat mycoplasma (2A)	45	12	321	n.d.	100
	DFMO	9	95	87	18	171
	Rat mycoplasma (682)	123	488	141	n.d.	84
	DFMO	108	572	76	21	108
	Bovine mycoplasma	21	17	194	n.d.	77
	DFMO	10	41	110	6	114
	<i>M. suipneumoniae</i>	66	9	289	n.d.	76
	DFMO	n.d.	17	51	7	141

decarboxylase activities, mycoplasma extracts promoted barely detectable decarboxylation of *S*-adenosylmethionine. Infection of growing cultures of Ehrlich ascites carcinoma cells and L1210 mouse leukaemia cells with the mycoplasmas resulted in a change of tumour cell polyamine pattern, the appearance of cadaverine being the most distinct feature (Table 3). It is noteworthy that, although cultures of *M. suipneumoniae* did not contain

cadaverine (Table 1), an infection of the tumour cells with this mycoplasma strain also brought about an accumulation of cadaverine in both cell lines (Table 3).
When the infected cells were exposed to 3 mM-2-difluoromethylornithine, the accumulation of cadaverine was distinctly enhanced and some aminopropylcadaverine was likewise formed (Table 3). In general, 2-difluoromethylornithine-treated infected

cells also contained more putrescine and spermidine than did uninfected cells. The 2-difluoromethylornithine-induced enhanced accumulation of cadaverine in mycoplasma-infected cells could be largely prevented by addition of micromolar concentrations of spermidine (results not shown).

Discussion

The distinct changes in the polyamine pattern, especially the appearance of cadaverine, in tumour cells after the infection with mycoplasmas could possibly be used as a diagnostic measure to uncover mycoplasma contamination in animal cell cultures. This is particularly important, since there are no entirely reliable morphological or metabolic indicators for hidden mycoplasma infections. Under our experimental conditions, growth inhibition produced by the infection with the different mycoplasmas was either absent or rather marginal, never constant. Nor did the microscopic appearance of the cells change on prolonged infection. The changes in the polyamine pattern could be further magnified by exposing the cultures to 2-difluoromethylornithine, a powerful inhibitor of putrescine and spermidine formation in eukaryotic cells (Metcalf *et al.*, 1978); this compound was ineffective with respect to polyamine synthesis in the mycoplasmas.

We recently reported that in one of our Ehrlich ascites carcinoma cell lines adapted to growth in suspension cultures, putrescine and spermidine depletion produced by 2-difluoromethylornithine resulted in a markedly enhanced formation of cadaverine and its aminopropyl derivative (Alhonen-Hongisto & Jänne, 1980). However, in the new adaptation batch of the carcinoma cells used in the present experiments, there were no signs whatsoever indicating cadaverine formation in either the absence or the presence of 2-difluoromethylornithine (see also Table 3). The possibility, of course, remains that the previously used cell line had undergone mutations resulting in the expression of an inducible lysine decarboxylase. The fact that we were unable to detect any lysine decarboxylase activity in tumour cell extracts *in vitro* (Alhonen-Hongisto & Jänne, 1980) makes the latter explanation somewhat unlikely. The markedly enhanced accumulation of cadaverine found in 2-difluoromethylornithine-treated cells (Alhonen-Hongisto & Jänne, 1980)

could simply be based on stimulated uptake of extracellular cadaverine, possibly produced by contaminating mycoplasmas, since polyamine depletion (by 2-difluoromethylornithine) greatly enhances the uptake of exogenous polyamines (Alhonen-Hongisto *et al.*, 1980) including cadaverine (Alhonen-Hongisto & Jänne, 1980).

Even though the previous observation (Alhonen-Hongisto & Jänne, 1980), suggesting the existence of an inducible lysine decarboxylase in tumour cells, may just be an artefact caused by hidden mycoplasma infection, the fact remains that the observed modifications of the intracellular polyamine pools by mycoplasmas in cultured animal cells can be taken as a warning sign indicating a contamination with this organism.

The excellent technical assistance of Ms. Merja Kärkkäinen and Raija Laine is gratefully acknowledged. This investigation received financial support from the National Research Council for Natural Sciences, Academy of Finland, and from the Finnish Foundation for Cancer Research.

References

- Alhonen-Hongisto, L. & Jänne, J. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1005–1013
- Alhonen-Hongisto, L., Pösö, H. & Jänne, J. (1979) *Biochim. Biophys. Acta* **564**, 473–487
- Alhonen-Hongisto, L., Seppänen, P. & Jänne, J. (1980) *Biochem. J.* **192**, 941–945
- Friis, N. F. (1969) *Acta Vet. Scand.* **10**, 295–297
- Friis, N. F. (1971) *Acta Vet. Scand.* **12**, 454–456
- Giles, K. & Myers, A. (1965) *Nature (London)* **206**, 93
- Hölttä, E., Jänne, J. & Pispa, J. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1165–1171
- Hölttä, E., Jänne, J. & Pispa, J. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1104–1111
- Jänne, J. & Williams-Ashman, H. G. (1971) *J. Biol. Chem.* **246**, 1725–1732
- Jänne, J., Pösö, H. & Raina, A. (1978) *Biochim. Biophys. Acta* **473**, 241–293
- Lowry, O. H., Rosebrough, N. J., Farr, A. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. & Vevert, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
- Rodwell, A. W. (1967) *Ann. N.Y. Acad. Sci.* **143**, 88–109
- Seiler, N. (1970) *Methods Biochem. Anal.* **18**, 259–337