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Effect of Inhibitors of Ornithine and Adenosylmethionine Decarboxylases on the Synthesis of Deoxyribonucleic Acid in Ehrlich Ascites Cells *in vivo*

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An inoculation of mice with Ehrlich ascites cells induced a rapid enhancement of polyamine synthesis in the tumor cells. The activities of L-ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase were substantially stimulated already 4 h after the intraperitoneal inoculation. The first peak of tumor DNA synthesis occurred around 20 h after the inoculation and was accompanied by significant increases in the concentration of cellular spermidine and spermine. Inhibition of increased ornithine decarboxylase activity after the inoculation by repeated doses (every 4 h) of 1,3-diaminopropane prevented any increases in tumor polyamines and likewise depressed the synthesis of DNA 20 h after the inoculation. The inhibition of DNA synthesis by diaminopropane was further increased when the amine treatment was continued for 40 h. DL-α-Difluoromethylornithine when given every 4 h produced a more rapid decrease in the concentration of cellular spermidine than did diaminopropane but increased cellular spermine and only insignificantly inhibited the synthesis of DNA. Less frequent injections of the compound (every 10 h) led to a marked decrease in spermidine content with only small increases in tumor spermine and clearly inhibited the synthesis of DNA in the ascites cells. A combination of diaminopropane of 1,3-diamino-2-propanol with *N*,*N*'-bis(3-aminoguanidino)-propan-1,2-diimine produced a striking synergism in decreasing the synthesis of DNA. The potentation of the antiproliferative effect of diaminopropane or diaminopropanol by *N*,*N*'-bis(3-aminoguanidino)propan-1,2-diimine appeared to be specific to the diamines since no such synergism was found when difluoromethyl ornithine and *N*,*N*'-bis(3-aminoguanidino)propan-1,2-diimine were injected together. Furthermore, the latter compound clearly slowed down the degradation rate of the diamines in the tumor cells.

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The recent few years have witnessed a remarkably rapid progress in research aimed to elucidate the physiological roles of the natural polyamines putrescine, spermidine and spermine. This has been possible mainly because of the discovery of a large number of compounds acting as more or less specific inhibitors of polyamine synthesis. Thus, practically for the first time, it has become possible to investigate the metabolic consequences of an intracellular depletion of polyamines. Most of the inhibitors of polyamine biosynthesis are congeners of L-ornithine or *S*-adenosyl-L-methionine intervening with the decarboxylation of these key amino acids, or they are indirect inhibitors of ornithine decarboxylase (EC 4.1.1.17) (ODC).[1]

In the majority of studies the use of inhibitors of polyamine biosynthesis, acting in different ways, has produced a clear antiproliferative effect with a profound depression of DNA synthesis/replication as a distinct feature.[1]

The causal relationship between polyamine depletion and the antiproliferative effect, although strongly suggestive, is not yet completely established especially since some of the drugs used as inhibitors may have other effects, possibly independently contributing to their antiproliferative action. Thus methylglyoxal bis(guanylhydrazone) (MGBG), a highly potent inhibitor of mammalian adenosylmethionine decarboxylase (EC 4.1.1.50) (AMDC),[2] prevents oxidative phosphorylation [3] and produces mitochondrial damage preceding its antiproliferative action.[4] MGBG likewise interferes with the

uptake of radioactive thymidine and inhibits its phosphorylation by thymidine kinase in lymphocytes,[5] effects which may or may not be related to the polyamine depletion. Furthermore, at least in certain circumstances the early antiproliferative effect of this compound can be dissociated from polyamine depletion.[6]

At this moment it appears that though more studies should be directed to the molecular mechanisms through which polyamine depletion produces its metabolic consequences, equally important is to collect more experimental data of the "universality" of these phenomena.

Ehrlich ascites carcinoma cells which contain substantial amounts of polyamines [7,8] and exhibit typical growth-dependent fluctuations in the activities of their biosynthetic decarboxylases [9,10] obviously offer a suitable model system for studies devoted to the elucidation of the role of polyamines in tumor cells. Of great importance was the observation [11] showing that ODC activity in Ehrlich ascites cells exhibited dramatic increases already 5 h after an inoculation of plateau-phase tumor cells with a second peak of enzyme activity emerging exactly at the time of the surge of the tumor cells from G_1 to S phase.[11]

We have now studied the changes of polyamine accumulation and synthesis during the very first days following the inoculation of Ehrlich ascites carcinoma cells into a new host. It appears that the inhibition of ODC by 1,3-diaminopropane (DAP), 1,3-diamino-2-propanol (DAP-OH) or by DL-difluoromethyl ornithine (DFMO), a recently discovered irreversible inhibitor of the enzyme,[12,13] results in a profound depression of DNA synthesis. Especially effective was the combination of DAP or DAP-OH with *N,N'*-bis(3-aminoguanidino)propan-1,2-diimine (MBAG), a derivative of MGBG which is not supposed to be antiproliferative alone.[14]

MATERIALS AND METHODS

Ehrlich ascites cells. Female albino mice (weighing about 30 g) were used in all experiments. The mice were inoculated with $6-8.4\times10^6$ cells/2 ml in physiological saline. In the experiment presented in Table 1 the animals received saline, putrescine (150 μmol/100 g) or 1,3-diaminopropane 150 μmol/100 g) as intraperitoneal injections every 4 h until killed 20 h after the inoculation. In the experiment of Table 2 the mice received saline, diaminopropane (150 μmol/100 g) or difluoromethylornithine (DFMO; 40 mg/100 g) every 4 h until killed 20 or 40 h after the inoculation. In the experiment 1 of Table 3 the mice received saline, diaminopropane (150 μmol/100 g), MBAG (2 mg/100 g) or the combination of the drugs at the time of the inoculation and 10 h thereafter, in experiment 2 of Table 3 diaminopropanol (100 μmol/100 g) was used instead of diaminopropane. In the experiment 3 of Table 3 the animals received difluoromethylornithine (DFMO; 40 mg/100 g) instead of diaminopropane and only DFMO was injected 10 h after the inoculation. In all the three experiments of Table 3 the animals were killed 20 h after the inoculation.

Chemicals. *N,N'*-bis(3-aminoguanidino)propan-1,2-diimine (MBAG) was synthesized by the method of Baiocchi *et al.*[14] using *N,N'*-diaminoguanidine·HCl (Aldrich-Europe, Beerse, Belgium) and methylglyoxal (Sigma, St. Louis, Mo.) as starting material. 1,3-Diaminopropane and 1,3-diamino-2-propanol were obtained from Fluka AG (Buchs, SG, Switzerland) and methylglyoxal bis(guanylhydrazone) (MGBG) from Aldrich-Europe. DL-Difluoromethylornithine was a generous gift from Centre de Recherche Merrell International (Strasbourg, France). DL-[1-^{14}C]Ornithine (specific radioactivity 59 Ci/mol) and [6-^{3}H]thymidine (specific radioactivity 26 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, Bucks., U. K.). *S*-Adenosyl-L-[1-^{14}C]methionine was synthesized enzymically as described in detail by Pegg and Williams-Ashman.[15]

Analytical methods. Ascites carcinoma cells were disintegrated ultrasonically and after centrifugation for 30 min at 100 000 g_{max} the activities of ornithine decarboxylase [11] and adenosylmethionine decarboxylase [17] were assayed by published methods. The synthesis of DNA *in vivo* was measured by injecting the mice with 10 μCi of [^{3}H]thymidine and killing the animals 5 min later. Under these conditions the incorporation of radioactive thymidine into cellular DNA was linear both regarding the time and the number of cells used. Putrescine, spermidine and spermine were measured electrophoretically by method of Raina and Cohen.[18] Diaminopropane was separated from putrescine as described earlier.[19] DNA was measured by the method of Giles and Myers [20] and protein by the method of Lowry *et al.*[21] The significance of the differences was calculated using two-tailed *t*-test.

RESULTS

Stimulation of tumor polyamine synthesis after inoculation. The inoculation of mice with a large number of plateau-phase ascites cells rapidly induced an increase in ODC activity in the tumor cells. The first peak of enzyme activity

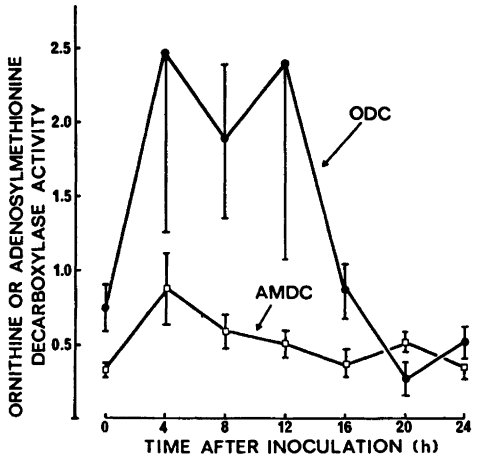

Fig. 1. Changes in ornithine decarboxylase (ODC) and adenosylmethionine decarboxylase (AMDC) activities in Ehrlich ascites cells following inoculation. The mice were inoculated with 2 ml of a cell suspension and killed at times indicated. There were 3 to 5 animals in each group. The vertical bars represent standard errors of the means. The enzyme activities are expressed as (nmol of CO_2 released)/(mg protein per 30 min).

occurred already 4 h following the injection of the tumor cell suspension (Fig. 1). The activity of AMDC was similarly, although not that markedly, enhanced within a few hours after the inoculation (Fig. 1). These results confirm a report of Harris *et al.*[11] showing that ODC activity of tumor cells is enhanced rapidly upon inoculation.

The stimulation of the biosynthetic enzymes of polyamines was accompanied by small, yet significant, elevations of tumor polyamine concentrations. The concentrations of all three polyamines were increased 4 h and spermidine as well as spermine exhibited a second rise 20 h after the inoculation (Fig. 2A). The synthesis of DNA *in vivo* steadily increased until 20 h after the inoculation (Fig. 2B). Thus the metabolism of ascites cells transferred into a new host was characterized by an early increase in the activities of ODC and AMDC with similar changes in cellular polyamine concentrations occurring earlier or at the same time as the stimulation of tumor DNA synthesis.

Effects of inhibitors of ornithine decarboxylase on polyamine accumulation and the synthesis of DNA in the tumor cells. We have shown earlier that the activity of ODC in Ehrlich ascites cells can be inhibited by a variety of physiological and unphysiological diamines.[10]

As shown in Table 1, repeated injections (every 4 h) of putrescine or 1,3-diaminopropane (DAP), starting at the time of the inoculation and continued for 20 h thereafter, virtually abolished any ODC activity in the ascites cells.

DAP also effectively decreased the accumulation of putrescine and spermidine while injections of putrescine, as expected, increased the content of putrescine and spermidine and marginally decreased the concentration of cellular spermine (Table 1). The moderate decrease in the concentrations of polyamines brought about by the injection of DAP was associated with more than 50 % inhibition of DNA synthesis, whereas similar doses of putrescine, which were equally effective in inhibiting ODC

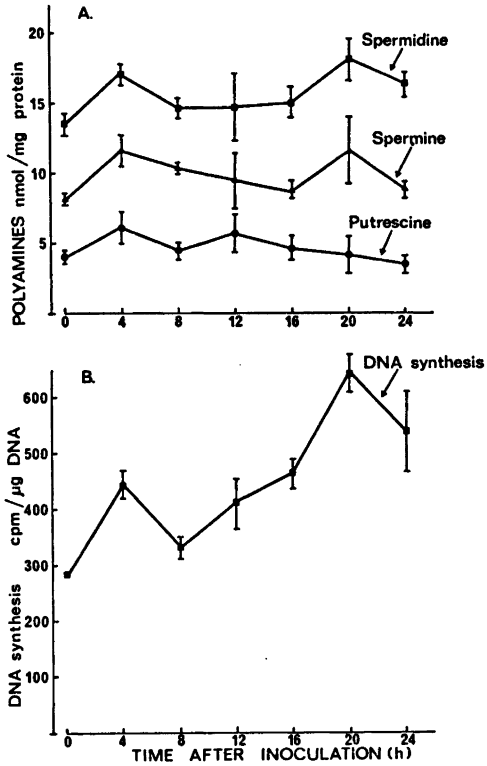

Fig. 2. Changes in polyamine concentrations (A) and DNA synthesis (B) in Ehrlich ascites cells following inoculation. For experimental details see the legend for Fig. 1.

Table 1. Effects of repeated treatments with putrescine and diaminopropane on the synthesis and accumulation of polyamines and DNA synthesis in Ehrlich ascites cells. Experimental details are given in materials and methods.

Treatment (Number of animals)	ODC activity nmol/mg protein	AMDC activity	Putrescine nmol/mg DNA	Spermidine	Spermine	DNA synthesis cpm/μg DNA
Saline (4)	1.25(33)	0.59(9)	60(4)	203(26)	149(8)	538(73)
Putrescine (4)	0.15(6) [a]	0.44(6)	96(7) [a]	258(9)	113(6) [a]	508(58)
Diamino-propane (4)	0.07(4) [a]	0.44(13)	25(6) [a]	130(24)	125(6) [b]	209(41) [a]

The means are given ± S.E.. The significances of the differences in comparison with the saline-treated group were: [a] $p < 0.01$, [b] $p < 0.05$.

activity, were without any effect on the incorporation of labelled thymidine into tumor DNA (Table 1).

If the treatment with DAP was continued for longer periods of time, *i.e.* for 40 h, the inhibition of DNA synthesis became more pronounced as did the decrease in the concentration of spermidine (Table 2). Table 2 likewise shows the effect of DFMO, a recently discovered irreversible inhibitor of ODC,[12,13] on polyamine accumulation and on the synthesis of DNA in Ehrlich ascites cells. This compound effectively decreased the concentration of tumor spermidine. When given every 4 h, a treatment lasting for 20 h was sufficient to decrease tumor spermidine content to less than 50 % of the control value and a treatment for 40 h resulted in the disappearance of more than 90 % of the original tumor spermidine (Table 2). Even though DFMO reduced the concentration of cellular spermidine much more effectively than did DAP the former compound only caused a marginal and statistically insignificant decrease in the synthesis of DNA as measured both 20 h and 40 h after the commencement of the treatment (Table 2). However, in contrast to the treatment with DAP, during which tumor spermine remained unchanged, DFMO treatment almost doubled the concentration of spermine at 40 h (Table 2). Apparently, the increase in the concentration of tumor cell spermine in DFMO-treated mice was due to a markedly enhanced AMDC activity, as was also noticed in hepatoma cells by Mamont *et al.*[12,13]

Table 2. Effects of repeated treatments with diaminopropane and difluoromethylornithine (DFMO) on polyamine accumulation and DNA synthesis in Ehrlich ascites cells.

Treatment (Number of animals)	Duration of treatment/h	Putrescine nmol/mg DNA	Spermidine	Spermine	Diamino-propane	DNA synthesis cpm/μg DNA
Saline (4)	20	25(3)	259(20)	124(12)	–	594(72)
Diamino-propane (5)	20	6(1) [a]	176(3) [b]	125(8)	172(37)	300(67) [b]
DFMO (5)	20	13(3) [c]	94(6) [a]	150(5)	–	491(18)
Saline (5)	40	24(2)	233(11)	139(5)	–	521(109)
Diamino-propane (5)	40	13(3) [c]	117(10) [a]	156(10)	239(34)	104(27) [b]
DFMO (4)	40	13(1) [c]	16(2) [a]	247(34) [c]	–	344(34)

The significance of the differences (as compared with the saline treated group) was: [a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$.

When normal tissue levels of putrescine are low this enzyme may function in the synthesis of spermine from spermidine. It is possible that under these circumstances the rapid drop in cellular spermidine in response to DFMO administration could partly be a result of an enhanced conversion of spermidine to spermine. The relative ineffectiveness of DFMO in decreasing DNA synthesis, in comparison with DAP, could thus be explained in terms of increased concentrations of spermine compensating the loss of spermidine. This idea was further supported by the experimental findings showing that when DFMO was given less frequently, *i.e.* only twice during the 20 h period, the compound still markedly decreased the concentration of spermidine but produced virtually no increase in spermine content (Table 3, exp. 3). Under these conditions the incorporation of [^{3}H]thymidine into tumor DNA was significantly inhibited (Table 3, exp. 3).

Combination of inhibitors of ornithine and adenosylmethionine decarboxylases. Some recent reports [22,23] have proposed that a proper combination of two inhibitors acting on different reactions of polyamine biosynthesis would result in a more profound antiproliferative effect at lower dose levels than when the inhibitors are used alone.

When MBAG, an irreversible inhibitor of AMDC,[24] was injected together with DAP a marked antiproliferative effect was achieved. Two injections of DAP alone did not have any effect on DNA synthesis, MBAG alone resulted in a small inhibition, while the combination of both drugs virtually abolished any incorporation of labelled thymidine into DNA (Table 3, exp. 1). The combination likewise slightly decreased cell mass. The mechanism of the striking synergism between DAP and MBAG is not known, although it appears that the latter compound inhibited the intracellular

Table 3. Effect of various inhibitors and their combination on ODC activity, accumulation of spermidine and spermine, DNA synthesis and cell mass in Ehrlich ascites cells.

Treatment (Number of animals)	ODC activity nmol/mg protein	Spermidine nmol/mg DNA	Spermine	DNA synthesis cpm/μg DNA	Cell mass g
Exp. 1.					
Saline (4)	0.48(10)	331(8)	181(7)	748(32)	0.64(3)
Diamino-propane (4)	0.69(7)	354(14)	229(9) ^{b}	705(32)	0.82(3)
MBAG (3)	0.27(2)	324(35)	187(9)	524(43)	0.64(4)
Diamino-propane +MBAG (4)	0.00(0) ^{a}	276(12) ^{a}	177(7)	36(16) ^{a}	0.49(1)
Exp. 2.					
Saline (8)	1.08(12)	367(17)	187(8)	2130(192)	0.82(3)
Diamino-propanol (8)	1.22(20)	340(9)	176(5)	2320(150)	0.88(4)
MBAG (8)	1.39(22)	349(19)	244(13) ^{b}	2400(283)	0.74(6)
Diamino-propanol +MBAG (7)	0.45(14) ^{b}	257(16) ^{a}	164(6) ^{c}	800(178) ^{a}	0.45(7) ^{a}
Exp. 3.					
Saline (5)	1.04(15)	312(23)	162(28)	861(28)	0.81(6)
DFMO (4)	1.06(21)	89(3), ^{a}	201(12)	515(28) ^{a}	0.79(4)
MBAG (5)	1.31(45)	326(16)	172(9)	986(97)	0.90(3)
DFMO +MBAG (5)	0.88(06)	138(8) ^{a}	208(9) ^{c}	601(54) ^{b}	0.73(4)

The significance of the differences, as compared with the saline treated groups, was: ^{a} $p < 0.001$, ^{b} $p < 0.01$, ^{c} $p < 0.05$.

degradation of the diamine. In fact, our results have indicated that MBAG, just like MGBG,[25] is an extremely potent inhibitor of diamine oxidase activity (using rat thymus as the source of the enzyme), a finding also noticed by others.[23,26] We noticed that DAP fraction, which was hardly detectable when the amine was injected alone, was clearly seen on the electropherograms after the treatment with the combination of DAP and MBAG although it was still not sufficient for reliable quantitation.

We next combined MBAG with 1,3-diamino-2-propanol (DAP-OH), a potent inhibitor of mammalian ODC,[27] using the same experimental design as in Table 3, exp. 1, except that the dose of DAP-OH was somewhat reduced in comparison to DAP. When the drugs were given separately there were only small changes in the activity of ODC, concentrations of polyamines or DNA synthesis (Table 3, exp. 2). The combination of both inhibitors again significantly decreased the concentrations of spermidine and spermine and also markedly inhibited the incorporation of labelled thymidine into DNA (Table 3, exp. 2). In addition there likewise was a significant decrease in the cell mass (Table 3, exp. 2).

As in the case of DAP, MBAG appeared to prevent the metabolism of DAP-OH, yet the concentrations were too low for a direct quantitation. However, the markedly decreased ODC activity seen after the combination of MBAG either with DAP (Table 3, exp. 1) or DAP-OH (Table 3, exp. 2) can also be taken as evidence for a prolonged action of the diamines in the presence of MBAG which itself is not inhibitory to the enzyme.

The idea that MBAG largely, if not necessarily exclusively, acts through a decrease in diamine degradation and possibly not that markedly through an inhibition of AMDC, was further supported by the experimental findings presented in Table 3, exp. 3. As shown in the table, two injections of DFMO produced a significant inhibition of DNA synthesis in the tumor cells while MBAG alone was ineffective. A combination of DFMO with MBAG, if anything, reduced the extent of the inhibition achieved with DFMO alone (Table 3, exp. 3). Similarly, MBAG did not potentiate the action of DFMO in decreasing the concentrations of spermidine and spermine.

DISCUSSION

Even though the molecular mechanism of polyamine action in cellular physiology remains to be determined, it appears obvious that a selective depletion of these compounds leads to an antiproliferative effect with depressed synthesis of DNA as the major feature.[1] This antiproliferative action due to polyamine depletion occurs both in animal and neoplastic cells, although the changes in polyamine synthesis in response to diverse growth stimuli may be greatly different in various cell types. In regenerating rodent liver, an early stimulation of ODC activity [28,29] results in a rapid accumulation of liver putrescine [30] followed by a gradual increase in the concentration of spermidine, while hepatic spermine content initially decreases.[31] In contrast to regenerating liver, the fluctuations of ODC in Ehrlich ascites cells (Fig. 1) appeared to be reflected more directly as changes in spermidine and spermine concentrations with little changes in the concentration of tumor putrescine. The latter finding implies that in ascites carcinoma cells spermine may play a more active role in cell proliferation than in the regenerating hepatocyte.

All the currently available inhibitors of polyamine biosynthesis have their own intrinsic disadvantages especially when used *in vivo*. Unphysiological diamines, such as DAP [32] or its derivatives,[27] require repeated administrations owing to their rapid metabolism. Moreover, their inhibitory action on ODC activity and putrescine accumulation may wear off when used for longer periods of time (Table 2, Wiegand and Pegg [23]). Diamines, even those not normally found in animal tissues, could conceivably take over the cellular functions of natural polyamines or, as recently shown, some of them can serve as precursors in the synthesis of higher polyamines through the transfer of the aminopropyl group from decarboxylated adenosylmethionine.[33]

DFMO, a recently discovered promising irreversible inhibitor of ODC,[34,12,13] is an effective compound which rapidly decreases the concentration of cellular spermidine. This compound, however, has the peculiar property of enhancing the accumulation of spermine when given in sufficient doses (Table 2). The accumulation of spermine in response to DFMO

administration is most likely based upon a stimulation of AMDC activity [12,13] which, in the absence of putrescine synthesis, rapidly results in an enhanced formation of spermine. It is possible that this kind of mechanism also contributes to the swift and selective disappearance of spermidine after treatment with DFMO.

MGBG, which is a potent inhibitor of mammalian AMDC, has been employed to produce a polyamine depletion in a variety of systems.[1] MGBG also has effects which may or may not be caused by the inhibition of the synthesis of higher polyamines. These include effects on mitochondrial functions (oxidative phosphorylation) [3] and morphology.[4] MGBG also produces a severalfold accumulation of cellular putrescine partly due to the block of spermidine synthesis and partly due to a stimulation of ODC activity.[25,35]

MBAG, a derivative of the latter compound, is an irreversible inhibitor of AMDC [24,36] and, unlike MGBG, is not reported to possess any antiproliferative effects alone.[14] In combination with DAP, or DAP-OH, but not with DFMO, this compound exhibited a profound inhibition of DNA synthesis both in Ehrlich ascites cells (Table 3) and in regenerating rat liver.[23] It is possible that the main action of MBAG is to slow down the intracellular degradation of diamines, such as DAP, and its effect on the activity of AMDC might be of minor importance. Similar conclusions have also been reached by Pegg *et al.*[37]

It appears to us that in further design of inhibitor combinations for polyamine biosynthesis one should take into consideration (i) the polyamine pattern and the activities of their biosynthetic enzymes in a given organism or cell type, (ii) the dose response curves for the inhibitors so as to avoid possible secondary effects that may nullify the overall effect of the compound and (iii) whether two inhibitors, seemingly acting at different sites of polyamine formation, do have a common site of action by influencing their mutual metabolism.

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