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Polyamines and Their Derivatives as Modulators in Growth and Differentiation

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The polyamines and their derivatives are essential for life in eukaryotic and most prokaryotic cells, but their exact role in preserving cell function is not clear. These polyamines provide endogenous cations and thus participate in regulation of the intracellular pH; in addition, polyamine derivatives modulate cell growth and differentiation. The naturally occurring monoacetyl derivatives can induce increased activity of ornithine decarboxylase, the first enzyme in polyamine synthesis, and thus produce positive feedback to their production. The diacetyl derivatives of putrescine and of the synthetic analogue, 1,6 diaminohexane, induce differentiation and inhibit growth in many types of cells *in vitro*. In addition, they inhibit the proliferative and secretory response of normal B lymphocytes to B-cell mitogens and reduce production of antibodies *in vitro*. They also inhibit the proliferation of chronic lymphocytic leukemia cells (a B-lymphocyte leukemia). The parent polyamines are post-translational modifiers of proteins, and hypusine, a derivative of spermidine, is a covalently bound constituent of the eukaryotic protein synthetic initiation factor, eIF-4D.

Although these various actions do not at present fall into a coherent pattern, they clearly indicate that polyamines and their derivatives play an important part in modulating cell proliferation and differentiation.

INTRODUCTION

Putrescine, spermidine, and spermine, collectively known as the polyamines, have recently been the focus of increasing research activity. They are essential for the life of all eukaryotic cells, since mutants lacking the ability to synthesize them die unless the polyamines are provided in cell culture medium [1]. These metabolically related polyamines carry a positive charge at physiological pH and are the most abundant endogenously produced cations, but this important function does not explain their essential role in cell survival. Our work as well as that of others has shown that these low molecular weight aliphatic amines are elevated in association with increased cellular activity and are implicated in a wide range of diverse cell functions [1–5]. Putrescine, the parent diamine, arises intracellularly by decarboxylation of the

Abbreviations: Anti-μ: immunobeads, anti-human IgM, B-cell mitogen CLL: chronic lymphocytic leukemia GABA: γ-aminobutyric acid HMBA: hexamethylene*bis*acetamide (N,N^1 diacetyldiaminohexane) HPLC: high-performance liquid chromatography LPS: lipopolysaccharide, B-cell mitogen ODC: ornithine decarboxylase PHA: phytohemagglutinin, T-cell mitogen SAC: formalinized *Staphylococcus aureus,* Cowan strain I, B-cell mitogen SDS: sodium dodecyl sulfate TMBA: tetramethylene*bis*acetamide (N,N^1 diacetylputrescine) 8-BrcGMP: 8-bromo-3′,5′-cyclic guanosine monophosphoric acid

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Putrescine: $H_3\overset{+}{N}(CH_2)_4\overset{+}{N}H_3$

Spermidine: $H_3\overset{+}{N}(CH_2)_4\overset{+}{N}H_2(CH_2)_3\overset{+}{N}H_3$

Spermine: $H_3\overset{+}{N}(CH_2)_3\overset{+}{N}H_2(CH_2)_4\overset{+}{N}H_2(CH_2)_3\overset{+}{N}H_3$

Monoacetylputrescine: $H_3CCONH(CH_2)_4\overset{+}{N}H_3$

N^8Acetylspermidine: $H_3CCONH(CH_2)_4\overset{+}{N}H_2(CH_2)_3\overset{+}{N}H_3$

N^1Acetylspermidine: $H_3\overset{+}{N}(CH_2)_4\overset{+}{N}H_2(CH_2)_3\overset{+}{N}HCOCH_3$

Diacetylputrescine: $H_3CCONH(CH_2)_4NHCOCH_3$
(tetramethylene*bis*acetamide, or TMBA)

An Analogue: $H_3CCONH(CH_2)_6NHCOCH_3$
(hexamethylene*bis*acetamide, or HMBA)

FIG. 1. The polyamines and some of their acetylated derivatives.

amino acid ornithine by the enzyme ornithine decarboxylase (ODC). Spermidine and spermine are formed from putrescine by the sequential addition of two aminopropyl moieties derived from methionine. Putrescine, spermidine, and spermine are collectively referred to as the parent polyamines. Both acetylated and oxidized forms of these compounds are known to occur naturally (Fig. 1).

In spite of their highly conserved and essential nature, the exact physiological functions of the polyamines are not clear. In addition to their role as intracellular cations, they are known to modulate the conformation and transcription of DNA (at least *in vitro*), and to participate in the post-translational modification of proteins. Our major interest, however, has been in the physiological function of their derivatives in cellular growth and differentiation. These derivatives include γ-aminobutyric acid (GABA) and the monoacetylated derivatives of the parent polyamines themselves [6–8]. Also, the N,N′ diacetyl derivative of putrescine (tetramethylene*bis*acetamide, or TMBA) can induce differentiation of many types of cells in culture [1,2,4,9–12], and modulate lymphocyte function [1,2,4,13–16].

The monoacetyl derivative of putrescine, the isomeric forms of monoacetylspermidine and the diacetyl derivatives of putrescine and 1,6 diaminohexane were required for the investigations. Both radioactive and non-radioactive compounds were chemically synthesized by methods providing unique and confirmed configurations of the derivatives [10,17]. Polyamines and their primary amino derivatives were measured by a high-performance liquid chromatographic (HPLC) system utilizing a cation exchange resin and post-column derivatization with orthophthalaldehyde [17]. Tritiated or ^{14}C-labeled compounds were quantitated by liquid scintillation counting. Post-translationally labeled protein derivatives were separated by HPLC molecular sieving [18–20].

OBSERVATIONS

Control of Ornithine Decarboxylase

The concentration of putrescine, the precursor of other polyamines and polyamine derivatives, is determined in large degree by the activity of ornithine decarboxylase,

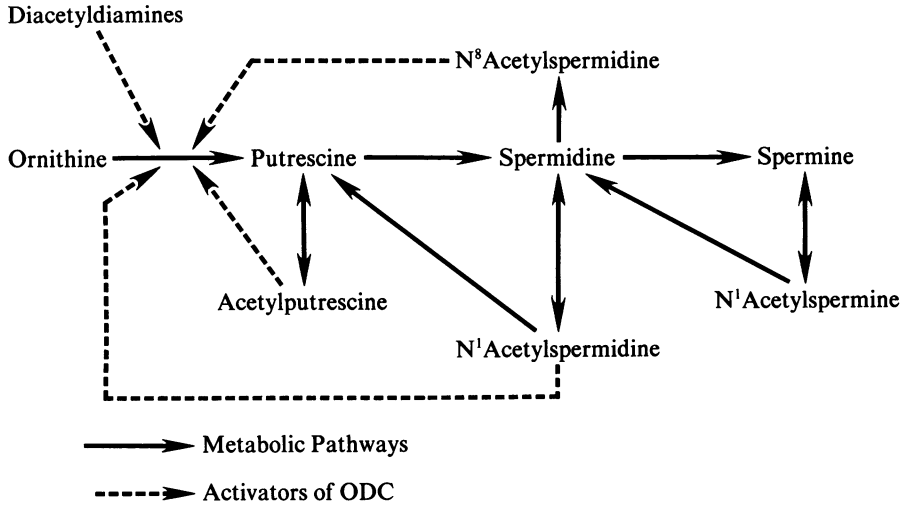

FIG. 2. Cycling of the polyamines. Solid arrows reflect metabolic pathways. Dashed arrows reflect stimulation of ornithine decarboxylase activity.

which is, in turn, a reflection both of the total amount of the enzyme present in the cell and the portion of the enzyme which is active. We were the first laboratory to demonstrate the net synthesis of ODC [21–24], the first and rate-limiting enzyme in the biosynthesis of the polyamines. Proof rested on a novel method of raising an antibody at a time that predated the general availability of monoclonal antibodies. This antibody made it possible to demonstrate a net increase in the quantity of ODC present. Synthesis of ODC proved to be increased by dibutyryl cyclic AMP [25], calcium [26], and by monoacetyl derivatives of the polyamines themselves [7,8]. Naturally occurring monoacetylputrescine and the two isomeric monoacetylspermidines participate in a feedback mechanism which regulates ODC [7,8]. The metabolic interrelationships between the parent polyamines and their acetylated derivatives permit cycling between these forms and maintenance of intracellular levels of each compound (Fig. 2). In each case (Table 1), the acetylated derivative, when added exogenously to cells in culture at very low levels, results in a large increase of ODC activity.

TABLE 1
Induction of Ornithine Decarboxylase by Acetylpolyamines

Compound	Concentration at Maximum Stimulation (M)	% of Unstimulated Level
N monoacetylputrescine	5×10^{-5}	361
N^1acetylspermidine	2.5×10^{-6}	742
N^8acetylspermidine	2.5×10^{-7}	496
HMBA	$5 \times 10^{-5} - 5 \times 10^{-6}$	510

Acetylpolyamines were added at various concentrations to HTC cells (Morris rat hepatoma) growing in log phase and ODC activity was measured enzymatically. The experimental results are expressed as percentage of control which measured ODC activity in simultaneous cultures [7, 8].

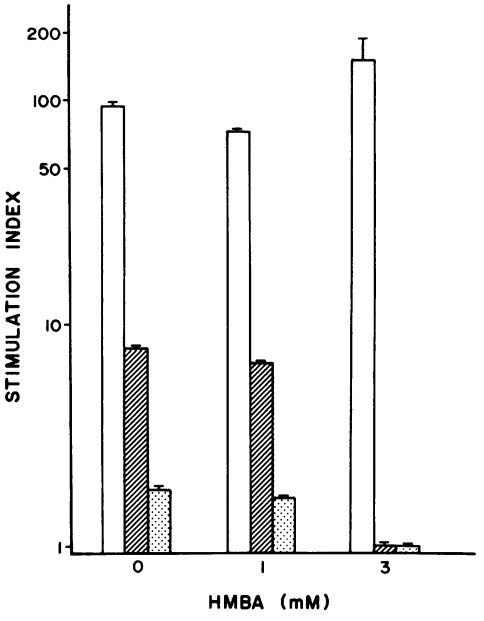

FIG. 3. BALB/c spleen cells were cultured for 72 hours at various concentrations of HMBA and with either PHA (1.0 μg/ml; *open bars*), phenol-extracted *E. coli* K235 LPS (50 μg/ml; *hatched bars*) or 8-BrcGMP (2 mM; *speckled bars*). Activation of proliferation was measured by ^{3}H-thymidine pulsing. Stimulation index is defined as $cpm_{exp}/cpm_{control}$ for ^{3}HTdR incorporation [13].

Effects of Diacetyldiamines

Diacetylputrescine and its chemical analogue, HMBA (hexamethylene*bis*acetamide, or diacetyl 1,6 diaminohexane) are immunomodulatory agents that exert effects selectively on B cells. They inhibit both proliferation and function of B lymphocytes.

In preliminary studies with mice, non-toxic intramuscular injection of HMBA yielded spleen cultures which were less capable of stimulation by B-cell mitogen than splenic lymphocytes from untreated animals. TMBA or HMBA, added exogenously to cells in culture, inhibits both proliferative capacity and immunoglobulin secretion. The effects of TMBA and HMBA are similar. Cultures of murine splenic lymphocytes [13,20] and of lymphocytes from normal human subjects and from patients with chronic lymphocytic leukemia (CLL) all show similar effects [14,15]. HMBA abrogates the proliferative response of cultured murine splenic lymphocytes to B-cell mitogens (lipopolysaccharide and 8-bromo-3′,5′-cyclic guanosine monophosphoric acid, or 8-BrcGMP) but not to a T-cell mitogen (phytohemagglutinin) ([13], Fig. 3). Immunoglobulin secretion is also profoundly decreased by HMBA ([13], Table 2). HMBA and TMBA also inhibit the proliferative response to B-cell mitogen in cultured lymphocytes from normal subjects ([14], Table 3). We sought to extend these observations to a study with clinical potential. Accordingly, we studied cells from patients with CLL, a disease which is characterized by a monoclonal expansion of B cells [15]. Again we noted that diacetyldiamine inhibits the proliferative capabilities of CLL cells in culture (Table 4). Thus, the effects of diacetyldiamines on lymphocytes are characteristically exhibited on B cells.

Timing of the Effect of Diacetyldiamine

The antiproliferative effect exerted by diacetyldiamine is associated with cell uptake of the diacetyldiamine. Inhibition of proliferation is observed even if diacetyldiamine is added as late as 48 hours after the start during a 72-hour incubation of stimulated

TABLE 2
Effects of Acetylated Polyamines on Immunoglobulin Secretion

	Number of Plaque-Forming Colonies Mean ± S.D.	
Conditions	Experiment 1	Experiment 2
LPS	205 ± 40	43 ± 15
LPS + HMBA	45 ± 10	10 ± 6

BALB/c spleen cells were cultured with 10 μg/ml of phenol-extracted *E. coli* K235 LPS in the absence or presence of 2 mM HMBA. The LPS-induced IgM plaque-forming response was measured after four days of culture [13].

murine splenic lymphocytes [13]. This effect occurred without loss of viability. TMBA persistently suppresses mitogenesis of human peripheral lymphocytes if it is preincubated with cells for 24 hours, and then incubation is continued for 72 hours more in the absence of TMBA ([14], Table 5). These findings suggest that diacetyldiamine becomes associated with the cell and continues to exert a persistent effect even after its removal from the cell's external environment.

Both TMBA and HMBA Are Taken Up by Cells in Culture and Metabolized

A portion of the diacetyldiamine that is taken up by the cell remains in its diacetylated form, a portion is present as the monoacetyl derivative, and a portion is hydrolyzed to the free diamine. Monoacetylputrescine (the partial hydrolysis product of TMBA) is found in association with the membrane fraction of cells [10, 11]. The level of monoacetylputrescine is significantly increased when differentiation of Friend erythroleukemia cells is induced with TMBA. During TMBA-induced differentiation of Friend erythroleukemia cells, monoacetylputrescine appears exclusively in the membrane fraction of cells [10,11]. This result suggests a specific and dynamic role for membranes in polyamine metabolism. When TMBA replaces dimethyl sulfoxide as a

TABLE 3
Inhibition of Proliferation by TMBA

Cell Culture	Inhibition by TMBA (%)
Control	11
Activated: anti-μ	82
Activated: SAC	75

B-cell enriched cultures purified from peripheral blood of normal donors were incubated for 72 hours either alone or in the presence of anti-μ (immunobeads, 30 μg antibody/ml) or SAC (formalinized *Staphylococcus aureus*, Cowan strain I, 0.01 percent v/v) and in the absence or presence of 4 mM TMBA. Proliferation was measured by thymidine pulsing. The effect of TMBA is expressed as the percentage of inhibition of proliferation caused by the addition of TMBA [14].

TABLE 4
Inhibition of Proliferation of Activated CLL Lymphocytes by HMBA

Patient	Cells/ml	% Inhibition
A	3×10^6	86
	2×10^6	89
	8×10^6	61
B	3×10^6	62
C	4×10^6	71
D	3×10^6	84
Human Splenic Lymphocytes: Small Cell Lymphocytic Lymphoma (B-cell IgD)	3×10^6	84

Cells obtained from patients with CLL were activated by SAC (formalinized *Staphylococcus aureus*, Cowan strain I, 0.01 percent v/v) and cultured for 72 hours in the absence or presence of 3 mM HMBA. Proliferation was measured by thymidine pulsing. The effect of HMBA is expressed as the percentage of inhibition of proliferation caused by the addition of HMBA [15].

differentiating agent with Friend erythroleukemia cells, there is a large increase in intracellular putrescine levels [11]. On the other hand, when HMBA is used as a differentiating agent, the putrescine levels fall dramatically, and there is a commensurate increase in levels of 1,6 diaminohexane (the hydrolysis product of HMBA); intracellular levels of 1,6 diaminohexane are equal to those attained by putrescine when TMBA is used as an inducing agent [11]. The increased cellular diamine concentrations are therefore derived from the diacetylated diamine, TMBA or HMBA, which is used as an inducing agent.

Diacetyldiamines Affect Cell Uptake of Polyamines

Exposure to diacetyldiamines decreases cell uptake of polyamines from the medium. A diminished uptake of radioactive putrescine or spermidine is consistently seen when

TABLE 5
Timing of the TMBA Effect

Cell Culture		Proliferation (% of control)
0–24 Hours	24–96 Hours	
Control	Control	100
Control	Anti-μ	733
Anti-μ	Anti-μ	802
Anti-μ and TMBA	Anti-μ	286
Control and TMBA	Anti-μ	357
Anti-μ and TMBA	Anti-μ and TMBA	191

B-cell enriched cultures purified from peripheral blood of normal donors were incubated for 24 hours either alone or in the presence of anti-μ (immunobeads, 30 μg/ml) or 4 mM TMBA as described above. After 24 hours of incubation the cells were washed, resuspended, and cultured with fresh additions as described above. Proliferation was measured by thymidine pulsing [14].

TABLE 6
Diacetyldiamines Affect the Uptake of Polyamines

Cells	Culture Conditions	Radioactive Polyamine (^{3}H–)	Inhibition of Uptake of ^{3}H-polyamine (% Inhibition)
Friend erythroleukemia cells:	Five-hour pulse:	Putrescine	
4 mM HMBA	Added at 5 hours		31
	24 hours		42
	72 hours		93
Murine spleen	Four-hour pulse: Added at 72 hours		
Control: 3 mM HMBA		Putrescine	90
		Spermidine	46
LPS activated: 3 mM HMBA		Putrescine	87
		Spermidine	80
Lymphocytes			
Normal human	48-hour incubation		
Control: 3 mM HMBA		Spermidine	37
SAC activated: 3 mM HMBA		Spermidine	67
CLL: 3 mM HMBA	72-hour incubation	Spermidine	42

Cells of various types in which we have demonstrated a specific effect of diacetyldiamines were incubated under culture conditions described above in the absence or presence of diactetyldiamine, and cell uptake of exogenous radioactive putrescine or spermidine was measured. In all cases, the radioactive polyamine was added to the exogenous medium at a level of 10 μCi/ml (0.30–0.05 μM). Cells were harvested, washed free of exogenous radioactivity, and cell-associated radioactivity was measured by scintillation counting. The effect of diacetyldiamine is expressed as the percentage of inhibition of uptake of exogenous radioactive polyamine caused by the addition of diacetyldiamine. Friend murine erythroleukemia cells (clone 19) induced to differentiate by HMBA were compared with their time-matched uninduced controls [11]. BALB/c murine spleen cells were cultured either as controls or activated by LPS (10 μg/ml) [20]. Purified lymphocytes from normal subjects or from patients with CLL were cultured either as controls or activated by SAC (*Staphylococcus aureus*, Cowan strain I 0.01 percent v/v) ([15] and research in progress).

cells are exposed to exogenous diacetyldiamines (Table 6). HMBA, at levels that modulate differentiation in Friend erythroleukemia cells or mitogenesis in lymphocytes, significantly depresses cell uptake of radioactive putrescine and spermidine. At an earlier period of incubation, less inhibition was seen when "pulses" of radioactivity were used than at a later time. It thus appears that, with time, uptake becomes progressively diminished.

Diacetyldiamines Affect the Metabolism of Intracellular Polyamines

Activation of lymphocytes is associated with active metabolism of putrescine (primarily to γ-aminobutyric acid and spermidine), whereas spermidine seems to be conserved [18]. A feature of the metabolism of spermidine in cultured lymphocytes is that they actively synthesized N^1acetylspermidine. Lymphocytes from normal subjects and from patients with CLL convert from 10 percent to 20 percent of total intracellular radioactivity derived from exogenous ^{3}H-spermidine to N^1acetylspermidine ([15], experiments in progress). When CLL cells are activated by B-cell mitogen, the conversion of spermidine to N^1acetylspermidine is enhanced. HMBA diminishes the conversion of spermidine to N^1acetylspermidine in CLL lymphocytes ([15], experiments in progress). In addition, embryogenesis is also associated with changes in

polyamine metabolism. As the embryo of the sea urchin (*Strongylocentrotus purpuratus*) develops, the percentage of conversion of spermidine to N^1acetylspermidine increases [27]. Thus, N^1acetylspermidine is a major, normal intracellular metabolite, and its increased biosynthesis seems to be associated with elevated cellular activity. This conversion of spermidine to N^1acetylspermidine, which is regulated by both mitogen activation and exposure of cells to exogenous diamine, is consistent with a cell modulatory role for N^1acetylspermidine.

Mitogen-activated murine spleen cells in culture have a demonstrable, albeit low, capacity to metabolize spermidine to N^1acetylspermidine [20]. By contrast, active biosynthesis of N^1acetylspermidine has not been observed in HTC cells [28], Friend erythroleukemia cells induced to differentiation [10,11], and cultured human foreskin keratinocytes [6]. Recently very low amounts of acetylspermidine have been reported in various normal tissues [29–32], tumor tissues [33,34], and in various drug-induced situations [35,36]. Both of the isomeric monoacetylspermidines have been found in urine, and the enzymes responsible for their biosynthesis are now well characterized [1]. In our studies with lymphocytes from various sources and the developing sea urchin embryo, we have detected only N^1acetylspermidine, and not N^8acetylspermidine.

Polyamines Are Modifiers of Proteins

Polyamines and their derivatives are present in proteins derived from cells of diverse cell types [1–4]. Labeled putrescine, spermidine, and spermine have been identified as covalently bound components of proteins in HTC cells which have been grown in medium containing radioactive putrescine [28]. Keratinocytes contain, in addition, large amounts of radioactive γ-aminobutyric acid as a primary protein-bound metabolite of exogenous putrescine [6]. In differentiating Friend erythroleukemia cells, hypusine is also present [11]. Hypusine is protein-bound lysine which has been post-translationally modified by the aminobutyl moiety of spermidine [37]. In the developing sea urchin embryo, radioactivity derived from exogenous spermidine accumulates in proteins in a stepwise manner which correlates with the cell cycle [38]. A unique 30 kD protein is present in very early embryogenesis [39]. As embryogenesis proceeds, the other polyamines as well as hypusine are present in several families of proteins which can be distinguished from each other by HPLC molecular sieving [18,37]. Several isoforms of the eukaryotic protein initiation factor eIF-4D which contains hypusine have also been identified [38].

Diacetyldiamines Affect the c-myc *Oncogene*

Activation of human B-lymphocytes is associated with an increased transcriptional expression of the c-*myc* gene [40]. Both TMBA and HMBA, at levels that suppress activation of these cells, also suppress c-*myc* mRNA [41]. These results suggest that the diacetylated diamines may play a regulatory role in B-cell activation by modulating expression of c-*myc*.

DISCUSSION

Although the mechanism by which polyamines and their derivatives affect cell function is not clear, certain facts are well established. Unless polyamines are present, cells do not grow or survive. The mechanisms for polyamine synthesis are highly conserved throughout all biological evolution. Whatever they do, therefore, polyamines must be important.

There is good evidence that the effects of polyamines are expressed through their derivatives. The monoacetyl derivatives are found at intracellular concentrations several orders of magnitude less than the millimolar concentrations of the parent polyamines. Through their monoacetyl derivatives, the polyamines exert control on the rate of synthesis of the parent compounds; the diacetyl derivatives of putrescine (and possibly other polyamines) affect the ability of cells to accumulate polyamines from the extracellular medium. It would be of interest to see whether other cations are also affected, but this question has not yet been studied.

In a large variety of cells, the diacetylated polyamines induce a change toward "maturation" in the sense that the cells change toward their mature phase. Erythroleukemia cells cease dividing and begin to make hemoglobin; B lymphocytes cease responding to stimuli to proliferation and antibody synthesis and remain in the terminal, relatively dormant mature phase.

The parent polyamines as well as γ-aminobutyric acid derived from putrescine are all covalently incorporated into proteins. Hypusine, which is derived from spermidine, is, to date, present in the only known protein derived from polyamines with a defined function; this protein plays a role in the cascade of events associated with protein biosynthesis. The pattern of labeling of proteins by polyamines and their metabolites suggests functions which remain to be clarified.

Many observers believe that acetylation of polyamines is important because it provides a suitable substrate for oxidases or facilitates export from the cell through obviation of cationic charges; however, our data show that the implications of polyamine acetylation are much broader. Thus, it appears that the derivatives of polyamines may be major factors through which the polyamines exert their physiological effects.

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