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Suppression of the Formation of Polyamines and Macromolecules by DL-α-Difluoromethylornithine and Methylglyoxal Bis(guanylhydrazone) in Phytohaemagglutinin-Activated Human Lymphocytes

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1. The activation of human peripheral blood lymphocytes by phytohaemagglutinin *in vitro* was accompanied by striking increases in the concentrations of the natural polyamines putrescine, spermidine and spermine. 2. The enhanced accumulation of polyamines could be almost totally abolished by DL-α-difluoromethylornithine, a newly discovered irreversible inhibitor of L-ornithine decarboxylase (EC 4.1.1.17), or by methylglyoxal bis(guanylhydrazone) {1,1′-[(methylethanediylidene)dinitrilo]diguanidine}, an inhibitor of *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50). The inhibition of polyamine accumulation was associated with a marked suppression of DNA synthesis, which was partially or totally reversed by low concentrations of exogenous putrescine, spermidine, spermine and cadaverine and by higher concentrations of 1,3-diaminopropane. 3. In contrast with some earlier studies, we found that methylglyoxal bis(guanylhydrazone), at concentrations that were sufficient to prevent polyamine accumulation, also caused a clear inhibition of protein synthesis in the activated lymphocytes. Similar results were obtained with difluoromethylornithine. The decrease in protein synthesis caused by both compounds preceded the impairment of DNA synthesis. The inhibition of protein synthesis by difluoromethylornithine was fully reversed by exogenous putrescine, spermidine and spermine, and that caused by methylglyoxal bis(guanylhydrazone) by spermidine and spermine. In further support of the idea that the inhibition of protein synthesis by these compounds was related to the polyamine depletion, we found that difluoromethylornithine caused a dose-dependent decrease in the incorporation of [^{14}C]leucine into lymphocyte proteins which closely correlated with the decreased concentrations of cellular spermidine. 4. Difluoromethylornithine and methylglyoxal bis(guanylhydrazone) also elicited a variable depression in the incorporation of [^{3}H]uridine and [^{14}C]adenine into total RNA. The apparent turnover of lymphocyte RNA remained essentially unchanged in spite of severe polyamine depletion brought about by difluoromethylornithine. 5. The present results, as well as confirming the anti-proliferative action of the inhibitors of polyamine biosynthesis, suggest that polyamine depletion may interfere with reactions at different levels of gene expression.

Enhanced synthesis and accumulation of the polyamines putrescine, spermidine and spermine appear to be invariably associated with cell growth and proliferation (Tabor & Tabor, 1976; Jänne *et al.*, 1978). A critical requirement of polyamines for normal cell multiplication is strongly suggested by the results obtained with polyamine-deficient mutants of micro-organisms (see Tabor & Tabor, 1976; Algranati & Goldemberg, 1977) and with eukaryotic cells specifically depleted in polyamines by use of inhibitors of their biosynthesis (see Jänne *et al.*, 1978). Various inhibitors of ornithine decarboxylase (EC 4.1.1.17) or adenosylmethionine decarboxylase (EC 4.1.1.50), chemically vastly different, have been shown to lead to a significant inhibition of growth and cell proliferation (Jänne *et al.*, 1978). Many reports support the view that the site of polyamine action is the synthesis of DNA itself, even though in some experimental systems the inhibition of polyamine synthesis also results in disturbances of protein and RNA synthesis (Kay & Pegg, 1973; Krokan & Eriksen, 1977). The picture is further complicated by the fact that some of the inhibitors appear to have effects that may or may not be mediated through the poly-

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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amine depletion. These include inhibition of oxidative phosphorylation and cell respiration (Pine & DiPaolo, 1966) and morphological damage of mitochondria by methylglyoxal bis(guanylhydrazone), changes that may precede its anti-proliferative action (Dave *et al.*, 1978).

Although an increased accumulation of polyamines has been shown to be an obligatory requirement for accelerated cell proliferation in a great variety of experimental systems, two major questions still remain unanswered: (i) what is (are) the exact site(s) of polyamine action in the cell cycle and (ii) do putrescine, spermidine and spermine have separate sites of action?

In the present study we have investigated the effects of difluoromethylornithine, a newly discovered irreversible inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978; Mamont *et al.*, 1978), and that of methylglyoxal bis(guanylhydrazone), a reversible inhibitor of adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972), on the formation of polyamines and macromolecules in phytohaemagglutinin-stimulated human lymphocytes. Both compounds caused a marked inhibition of polyamine accumulation, which was accompanied by a decreased rate of DNA synthesis. This inhibition of DNA synthesis was preceded by a marked decrease in the incorporation of labelled leucine into lymphocyte protein.

Experimental

Chemicals

Purified phytohaemagglutinin (type HA 16) was obtained from Wellcome Research Laboratories (Beckenham, Kent, U.K.). L-[U-^{14}C]Leucine (specific radioactivity 354mCi/mmol), [6-^{3}H]thymidine (specific radioactivity 26.4Ci/mmol), [5-^{3}H]uridine (specific radioactivity 27Ci/mmol), [8-^{14}C]adenine (specific radioactivity 54.2mCi/mmol), [U-^{14}C]glucose (specific radioactivity 304mCi/mmol) and [6-^{14}C]glucose (specific radioactivity 53.2mCi/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Putrescine, spermidine and spermine were obtained from Calbiochem (San Diego, CA, U.S.A.) and 1,3-diaminopropane was from Fluka A.G. (Buchs SG, Switzerland). Methylglyoxal bis-(guanylhydrazone) was purchased from Aldrich Europe (Beerse, Belgium). DL-α-Difluoromethylornithine was generously given by the Centre de Recherche Merrell International (Strasbourg, France).

Cell cultures

Human peripheral blood lymphocytes were purified from the buffy-coat fractions kindly supplied by the Finnish Red Cross Blood Transfusion Laboratory (Helsinki, Finland). A modification of the Ficoll/Isopaque centrifugation technique (Böyum, 1968) was used as described earlier (Hovi *et al.*, 1976). The resulting mononuclear leucocyte suspensions containing both lymphocytes and monocytes were cultured without further purification. The cells (usually 2×10^{6}/ml) were incubated in 60mm-diameter plastic Petri dishes in 5ml of RPMI 1640 medium (Gibco Bio-cult, Glasgow, Scotland, U.K.) supplemented with 10% dialysed pooled human serum and with G-penicillin (100i.u./ml) and streptomycin sulphate (50μg/ml). All solutions added to the cultures were sterilized by filtration through 0.22μm Millipore filters.

Analysis of polyamines

Lymphocytes were harvested by centrifugation at 1500*g* for 3min. The pellet was carefully drained and extracted twice with 0.1ml of 0.2M-$HClO_4$. The extracted polyamines were dansylated by the method of Seiler (1970) with the modifications described by Dreyfuss *et al.* (1973). The amount of dansyl chloride was decreased to 10mg/ml. Dansylated derivatives of the amines were separated on aluminium oxide thin-layer plates in chloroform/butan-1-ol (49:1, v/v) or chloroform/dioxan/butan-1-ol (48:1:1, by vol.). The fractions were localized under u.v. light, cut out, extracted with ethanol, and fluorescence intensity of the samples was determined with a Hitachi MPF-2 A fluorescence spectrophotometer.

Analysis of RNA and DNA

The precipitate remaining after $HClO_4$ extraction was washed with 1ml of 5% (w/v) trichloroacetic acid. The content of RNA was measured after alkaline hydrolysis (Ashwell, 1957). The remaining precipitate after RNA hydrolysis was subjected to acid hydrolysis in 5% trichloroacetic acid (90°C for 20min), and the content of DNA was measured by the method of Giles & Myers (1965).

The acid-insoluble material after DNA hydrolysis was dissolved in 0.1M-NaOH and used for protein measurement by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

When the rates of macromolecule synthesis were studied, the cell suspensions were labelled for 60 or 120min with [^{3}H]thymidine (0.2μCi/ml), [^{14}C]-leucine (0.1μCi/ml) and [^{3}H]uridine (0.2–1.6μCi/ml) or [^{14}C]adenine (0.05μCi/ml). Radioactive thymidine and uridine were diluted with unlabelled nucleosides to give a final concentration of 1μM. When three labelled precursors were used in the same experiment, one culture was labelled with [^{3}H]thymidine and [^{14}C]leucine, and another parallel culture with [^{3}H]uridine. The incorporation of radioactive precursors was halted by cooling the cell suspensions in

ice. The separation of RNA, DNA and protein was carried out as described above, except that an extra wash of the pellet was included at each stage of the fractionation. A portion of the fractions was analysed for radioactivity in Insta-Gel (Packard Instrument Co., Downer's Grove, IL, U.S.A.) in an LKB–Wallac liquid-scintillation counter.

Determination of thymidine kinase activity

Lymphocytes (10^7 cells) were suspended in 0.75 ml of 25 mM-Tris/HCl buffer (pH 7.4), containing 1 mM-dithiothreitol, and disintegrated by sonication 3 times for 5 s with a Branson sonifier. Thymidine kinase (EC 2.7.1.75) activity in the cytosol fraction was assayed by the method of Munch-Petersen & Tyrsted (1977).

Determination of glucose oxidation

The rate of glycolysis and the oxidation of glucose into CO_2 was measured by incubating the lymphocytes in the presence of [U-^{14}C]glucose (5 μCi/ml) or [6-^{14}C]glucose (2 μCi/ml) in small flasks equipped with a CO_2-trapping well. The evolved $^{14}CO_2$ was collected in 0.1 ml of Soluene-100 (Packard) and counted for radioactivity. The formation of radioactive lactate was determined by the method of Hoskins & Patterson (1968).

Results

Synthesis of macromolecules and accumulation of polyamines during lymphocyte activation

Under the experimental conditions used the maximum rate of DNA synthesis, as judged by the incorporation of [^{3}H]thymidine into the nucleic acid, occurred on the third day of lymphocyte activation in response to phytohaemagglutinin (Fig. 1*a*). The onset of DNA and RNA synthesis appeared to be preceded by an enhanced incorporation of labelled leucine into lymphocyte protein at the time (12 h) when no increase in the polyamine content was detected (Fig. 1). The concentrations of putrescine and spermidine were clearly elevated at 24 h after the stimulation, whereas the accumulation of spermine became evident somewhat later (Fig. 1*b*). As illustrated in Fig. 1(*b*), 72 h after phytohaemagglutinin administration the concentration of spermidine was increased 20-fold, and that of putrescine and spermine about 5-fold compared with the unactivated cells.

Effect of difluoromethylornithine and methylglyoxal bis(guanylhydrazone) on polyamine accumulation and DNA synthesis in activated lymphocytes

When 0.5 mM-difluoromethylornithine or 2.5 μM-

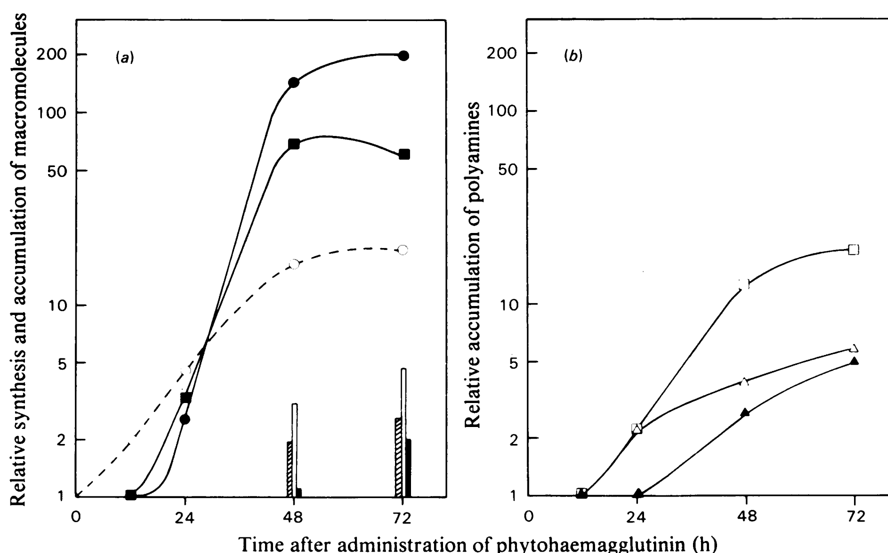

Fig. 1. *Stimulation of the synthesis and accumulation of DNA, RNA and protein (a) and the accumulation of polyamines (b) in human lymphocytes in response to phytohaemagglutinin administration*

The incorporation of [^{3}H]thymidine (●), [^{3}H]uridine (■) and [^{14}C]leucine (○) into DNA, RNA and protein was measured as described in the text at 12, 24, 48 and 72 h after phytohaemagglutinin (*a*). The amounts of protein (▨), RNA (□) and DNA (■) at 48 and 72 h after the stimulation are indicated as columns. The time course of the accumulation of spermidine (□), putrescine (△) and spermine (▲) after phytohaemagglutinin is shown in (*b*). The values are relative to those of the unstimulated cells at equivalent times.

methylglyoxal bis(guanylhydrazone) was added simultaneously with phytohaemagglutinin to the cultures, the enhanced accumulation of spermidine and spermine normally occurring after the stimulus was largely abolished (Table 1). Concomitantly, the incorporation of radioactive thymidine into DNA and the accumulation of cellular DNA were markedly inhibited (Table 1). Difluoromethylornithine appeared to decrease the concentration of spermidine more than did methylglyoxal bis(guanylhydrazone), whereas the latter compound more efficiently blocked the accumulation of spermine. The synthesis of putrescine, on the other hand, was strikingly stimulated by methylglyoxal bis(guanylhydrazone) and inhibited by difluoromethylornithine (Table 1). The combination of both inhibitors resulted in a further decrease in DNA synthesis and accumulation. Putrescine partially, and the higher polyamines totally, reversed the inhibition of DNA synthesis brought about by this combination (Table 1). The inhibition of DNA synthesis caused by difluoromethylornithine was fully reversed by putrescine, and also partially by low concentrations (10 μM) of cadaverine and higher concentrations (100 μM) of 1,3-diaminopropane. 1,2-Diaminoethane and 1,6-diaminohexane were ineffective at low concentrations.

Although 1,3-diaminopropane itself is an effective inhibitor of ornithine decarboxylase and can prevent the accumulation of putrescine and spermidine and the increase in DNA synthesis in regenerating liver (Pösö & Jänne, 1976), this diamine (at concentrations from 0.01 to 1 mM) only slightly prevented the accumulation of spermidine and did not have any effect on the accumulation of DNA or protein after phytohaemagglutinin stimulation (Table 2). Even though lymphocyte incubation in the presence of 2 mM-diaminopropane further decreased the polyamine concentration, the synthesis and accumulation of DNA were not disturbed. Only at high concentrations (10 mM) did diaminopropane lower the incorporation of thymidine into DNA (results not shown). It is possible that the ineffectiveness of diaminopropane in decreasing the synthesis of DNA in this

Table 1. *Association of polyamines with the synthesis of DNA in human lymphocytes*

Human lymphocytes (1×10^7) were stimulated with phytohaemagglutinin (PHA, 2 μg/ml) for 72 h. α-Difluoromethylornithine (DFMO, 0.5 mM) and methylglyoxal bis(guanylhydrazone) (MGBG, 2.5 μM) were added together with phytohaemagglutinin, whereas putrescine, spermidine and spermine, all at 10 μM, were added 17 h later. Incorporation of [^{3}H]thymidine into DNA was determined during the final 4 h of culture. All values are expressed per culture. Radioautographic analysis of the fraction of cells in the S-phase gave results that correlated well with the indicated thymidine-incorporation values.

Additions	[^{3}H]Thymidine incorporation (c.p.m.)	DNA (mg)	Putrescine (nmol)	Spermidine (nmol)	Spermine (nmol)
Control	940	0.030	0.12	0.25	0.97
PHA	254000	0.072	0.56	9.00	9.44
PHA+DFMO	66200	0.040	0.23	0.32	1.82
PHA+MGBG	43500	0.033	8.56	1.37	1.28
PHA+DFMO+MGBG	33600	0.030	0.35	0.76	1.50
PHA+DFMO+MGBG+putrescine	159000	0.043	19.40	3.25	1.49
PHA+DFMO+MGBG+spermidine	281000	0.069	0.25	14.10	7.81
PHA+DFMO+MGBG+spermine	287000	0.062	0.23	0.68	10.10

Table 2. *Effect of diaminopropane and difluoromethylornithine on the phytohaemagglutinin-induced accumulation of DNA, protein and polyamines*

Lymphocytes were incubated with phytohaemagglutinin (PHA, 2 μg/ml) with or without diaminopropane (DAP) or difluoromethylornithine (DFMO) for 72 h, whereafter the concentrations of DNA, protein and amines were determined as described in the Experimental section.

			Amount (nmol)			
Additions	DNA (mg)	Protein (mg)	DAP	Putrescine	Spermidine	Spermine
Control	0.025	0.176	—	0.10	0.23	0.75
PHA	0.045	0.612	—	0.25	6.87	9.25
PHA+DAP (0.01 mM)	0.044	0.663	1.95	0.16	5.10	7.00
PHA+DAP (0.1 mM)	0.048	0.688	7.80	0.15	5.30	8.75
PHA+DAP (1.0 mM)	0.052	0.694	70.00	0.10	5.30	4.20
PHA+DFMO (1.0 mM)	0.028	0.309	—	0.12	0.18	1.60

system may be due to its ability to take over some of the functions of the natural polyamines (see above).

In addition to the decreased incorporation of thymidine into DNA by difluoromethylornithine and methylglyoxal bis(guanylhydrazone), the compounds also inhibited thymidine kinase activity, which normally is greatly stimulated in response to phytohaemagglutinin addition (Table 3), in a manner closely correlated with DNA synthesis (Munch-Petersen & Tyrsted, 1977).

Table 3. *Effect of difluoromethylornithine and methylglyoxal bis(guanylhydrazone) on the activity of thymidine kinase in phytohaemagglutinin-stimulated lymphocytes*
Lymphocytes were stimulated with phytohaemagglutinin (PHA, 2 μg/ml) in the absence or presence of difluoromethylornithine (DFMO, 1 mM) or methylglyoxal bis(guanylhydrazone) (MGBG, 5 μM) for 48 h. For the determination of the activity of thymidine kinase see the Experimental section.

Additions	Activity of thymidine kinase (pmol/30 min) per mg of DNA	per mg of soluble protein
Control	620	150
PHA	37300	6660
PHA+DFMO	16700	3250
PHA+MGBG	22600	4010

Effect of polyamine inhibitors on the synthesis and accumulation of RNA and protein in activated lymphocytes

The inclusion of difluoromethylornithine and methylglyoxal bis(guanylhydrazone) in the lymphocyte cultures together with phytohaemagglutinin, as well as preventing polyamine accumulation and decreasing DNA synthesis, also invariably produced a pronounced decrease in the incorporation of [^{14}C]leucine into total protein (Table 4). Difluoromethylornithine and especially methylglyoxal bis(guanylhydrazone) likewise decreased the incorporation of radioactive uridine into RNA, even though this effect was less constant and usually not as marked as in Table 4. It is noteworthy, however, that both difluoromethylornithine and methylglyoxal bis(guanylhydrazone) markedly inhibited the accumulation of total RNA in response to phytohaemagglutinin stimulation for 72 h (Table 4). A simple labelling of RNA with radioactive uridine may give erroneous results of the synthetic rate of RNA, since a prolonged exposure of the lymphocytes to difluoromethylornithine may increase the uptake of [^{3}H]uridine into the cells as described below. The view that the inhibitory action of the two compounds on protein and RNA synthesis was mediated through polyamine depletion was strongly supported by the finding that exogenous polyamines largely reversed the inhibition (Table 4). From these experiments it is

Table 4. *Involvement of polyamines in the synthesis of macromolecules in human lymphocytes*
The experimental conditions were as described in Table 1, except for the concentrations of difluoromethylornithine (DFMO, 1 mM), methylglyoxal bis(guanylhydrazone) (MGBG, 5 μM) and exogenous polyamines (5 μM). Incorporation of the radioactive precursors into the macromolecules were determined during the final 2 h of culture. The results are the means of two cultures.

Additions	[^{3}H]Thymidine incorporation (c.p.m.)	[^{14}C]Leucine incorporation (c.p.m.)	[^{3}H]Uridine incorporation (c.p.m.)	DNA (mg)	RNA (mg)	Protein (mg)	Putrescine (nmol)	Spermidine (nmol)	Spermine (nmol)
Control	280	850	350	0.024	0.007	0.26	0.16	0.30	0.89
PHA	101000	26400	42800	0.061	0.098	0.99	0.47	8.25	11.00
PHA+DFMO	37300	6170	24800	0.030	0.021	0.45	0.27	0.30	1.76
PHA+DFMO+ putrescine	100000	22300	41700	0.064	0.091	1.01	0.67	7.63	13.20
PHA+DFMO+ spermidine	108000	26700	19700	0.066	0.083	1.02	0.43	7.65	11.00
PHA+DFMO+ spermine	119000	26900	43100	0.061	0.088	0.96	0.30	0.76	14.10
PHA+MGBG	21800	3120	6450	0.026	0.008	0.35	3.90	1.30	1.26
PHA+MGBG+ putrescine	78700	11800	39100	0.037	0.030	0.59	17.90	4.28	1.89
PHA+MGBG+ spermidine	108000	23900	39700	0.061	0.089	1.03	0.75	8.50	6.13
PHA+MGBG+ spermine	108000	26600	44100	0.059	0.086	1.01	1.20	3.53	14.10
PHA+DFMO+ MGBG	15200	2160	5370	0.025	0.008	0.33	0.22	0.30	1.36

exceedingly difficult to elucidate whether each polyamine exerts different effects in bringing about resumption of cell proliferation, since rapid conversion of putrescine into spermidine and spermine and of spermidine into spermine occurs (Table 4). Exogenous spermine was only slightly converted into spermidine, yet this polyamine fully reversed the inhibition of DNA, protein and RNA synthesis (Table 4).

The anti-proliferative action of methylglyoxal bis(guanylhydrazone) was most probably not due to the cytotoxic effects of the drug (Pine & DiPaolo, 1966; Dave *et al.*, 1978), since the formation of CO_2 and lactate from [U-^{14}C]glucose or [6-^{14}C]glucose was not decreased by more than 10% when 5 μM-methylglyoxal bis(guanylhydrazone) was present in phytohaemagglutinin-stimulated lymphocytes for 24h (results not shown).

Interestingly, as early as 24h after the stimulation of lymphocytes with phytohaemagglutinin, the incorporation of the labelled leucine into protein was distinctly (37%) inhibited by difluoromethylornithine and methylglyoxal bis(guanylhydrazone), whereas the initial increase in DNA synthesis was not affected by the drugs to any appreciable extent (Table 5). Furthermore, after 48h of stimulation the inhibition of protein synthesis by the drugs was greater than that of DNA synthesis (Table 5). At 48h the ratio of the protein to DNA was clearly decreased in cultures treated with the inhibitors (Table 5).

When difluoromethylornithine was added at increasing concentrations (0.01–5mM), the inhibition of protein synthesis exhibited a good correlation with the decrease in spermidine concentrations (Table 6). The maximum inhibition (about 50%) of leucine incorporation was achieved in the presence of 1mM-difluoromethylornithine, whereafter an increase in the drug concentration did not increase the inhibition (Table 6).

The incorporation of [^{3}H]uridine and [^{14}C]-adenine into RNA was also slightly inhibited by difluoromethylornithine and methylglyoxal bis-(guanylhydrazone) after 20h of lymphocyte activation, when no increase in DNA synthesis was observed (Table 7). At 48h, the rate of RNA synthesis was markedly inhibited by both inhibitors (Table 5).

Table 5. *Inhibition of polyamine and macromolecule formation by difluoromethylornithine and methylglyoxal bis(guanylhydrazone) during the course of lymphocyte stimulation by phytohaemagglutinin*

Lymphocytes were incubated with phytohaemagglutinin (PHA) in the absence or presence of difluoromethylornithine (DFMO) or methylglyoxal bis(guanylhydrazone) (MGBG) for the indicated times. For other details see Table 4. The results are normalized per DNA content of cultures, as the amount of DNA was not increased by more than 10–15% after lymphocyte stimulation for 48h.

Incubation (h)	Additions	[^{3}H]Thymidine incorporation (c.p.m.)	[^{14}C]Leucine incorporation (c.p.m.)	[^{3}H]Uridine incorporation (c.p.m.)	Protein/DNA	Putrescine (nmol)	Spermidine (nmol)	Spermine (nmol)
24	Control	420	1100	240	12.3	0.26	0.72	2.57
24	PHA	1070	4960	800	13.0	0.58	1.63	2.50
24	PHA+DFMO	1020	3100	1030	12.9	0.31	0.41	2.57
24	PHA+MGBG	950	3130	920	12.7	2.08	0.96	2.67
48	Control	470	1400	420	12.6	0.20	0.54	2.51
48	PHA	63900	21900	25600	23.0	0.77	5.69	6.75
48	PHA+DFMO	54400	9710	10800	18.5	0.19	0.27	3.82
48	PHA+MGBG	40300	8660	4100	18.5	4.01	1.60	2.51

Table 6. *Dose-dependence of the difluoromethylornithine-induced decrease in protein synthesis and spermidine level in phytohaemagglutinin-stimulated human lymphocytes*

Lymphocytes were stimulated with phytohaemagglutinin in the absence or presence of various concentrations of difluoromethylornithine (DFMO) for 48h. For experimental details and expression of data see Table 4.

Additions	Concentration of DFMO (mM)	[^{14}C]Leucine incorporation (c.p.m.)	Spermidine (nmol)
Control		1840	0.36
PHA		19800	4.13
PHA+DFMO	0.01	21600	5.30
PHA+DFMO	0.1	18900	1.15
PHA+DFMO	0.33	15200	0.57
PHA+DFMO	1	10500	0.38
PHA+DFMO	5	10300	0.38

Table 7. *Inhibition of the incorporation of [^{3}H]uridine and [^{14}C]adenine into RNA by difluoromethylornithine and methylglyoxal bis(guanylhydrazone) in phytohaemagglutinin-stimulated human lymphocytes before the onset of DNA synthesis*
Lymphocytes were incubated with phytohaemagglutinin in the absence or presence of difluoromethylornithine (DFMO, 1 mM) or methylglyoxal bis(guanylhydrazone) (MGBG, 5 μM) for 20 h. The incorporation of labelled uridine (8 μCi, 1 μM) or adenine (0.25 μCi, 1 μM) into RNA and DNA was determined during the last 60 min of culture.

	[^{3}H]Uridine incorporation into:		[^{14}C]Adenine incorporation into:	
Additions	RNA (c.p.m.)	Acid-soluble fraction (c.p.m.)	RNA (c.p.m.)	DNA (c.p.m.)
Control	1400	21300	2230	245
PHA	2490	34900	4280	235
PHA+DFMO	1780	37200	3420	210
PHA+MGBG	2070	41200	3400	215

A decrease in the concentration of RNA was observed after treatment for 2 days with methylglyoxal bis(guanylhydrazone) but not with difluoromethylornithine (results not shown).

Effect of polyamine depletion on the turnover of RNA

For the study of RNA degradation the cell cultures (incubated with phytohaemagglutinin in the absence or presence of 1 mM-difluoromethylornithine for 68 h) were labelled with [^{3}H]uridine (8 μCi, 1 μM) for 1 h, whereafter the pulses were chased with actinomycin D (5 μg/ml) and 100-fold excess of unlabelled uridine. The decay of radioactivity in RNA during the first 120 min revealed that the turnover of total RNA was not markedly influenced by the severe depletion of polyamines after treatment with difluoromethylornithine. The initial rate of RNA degradation, however, might be slightly higher in the polyamine-deficient cells than in the control cells (results not shown). It is noteworthy that the incorporation of [^{3}H]uridine into RNA could be even higher in the difluoromethylornithine-treated cells than in the stimulated cells without the inhibitor. This paradoxically high labelling of RNA after prolonged treatment of the lymphocytes with the inhibitor could be explained in terms of an increase in the label in the acid-soluble fraction of the cells (results not shown). Whether the polyamine deficiency results in an altered cell membrane permeability and increased uptake of the nucleoside remains to be determined.

Discussion

Lymphocyte transformation by phytohaemagglutinin has been reported to induce sequential increases in the synthesis of RNA, protein and DNA (Kay, 1968; Hausen *et al.*, 1969). However, according to a report on concanavalin A-activated lymphocytes from bovine lymph nodes, the synthesis of proteins is increased before the transcriptional activation (Hauser *et al.*, 1978). In the present experiments, an increase in the rate of protein synthesis was detected 12 h after the stimulation, when both RNA and DNA synthesis remained unchanged. A strikingly enhanced synthesis and accumulation of polyamines appear to be integrally associated with the activation of quiescent lymphocytes (Kay & Lindsay, 1973; Fillingame & Morris, 1973). In a careful study, Fillingame & Morris (1973) found that inhibition of polyamine accumulation by methylglyoxal bis(guanylhydrazone) in concanavalin A-activated bovine lymphocytes did not interfere with the processing or accumulation of the major species of RNA, nor was the synthesis of protein disturbed by the drug. They later reported that the treatment with methylglyoxal bis(guanylhydrazone) resulted in a selective inhibition of DNA synthesis and replication (Fillingame *et al.*, 1975; Morris *et al.*, 1977).

However, the present data do not support the concept that an inhibition of DNA synthesis is the only disturbance in macromolecular synthesis, at least as far as peripheral human lymphocytes are concerned. Our experimental results quite convincingly show that a distinct inhibition of protein synthesis precedes that of DNA synthesis in cultures where polyamine accumulation is prevented. It is even possible that the observed inhibition of thymidine incorporation into DNA in polyamine-depleted cells was partly due to a decreased thymidine kinase activity. This idea was advanced by Otani *et al.* (1977) on the basis of their studies on phytohaemagglutinin-induced lymphocytes after treatment with methylglyoxal bis(guanylhydrazone). A rapid decrease in the amount of thymidine kinase, possessing a relatively short molecular half-life (Barlow & Ord, 1975), is expected after inhibition of general protein synthesis. Moreover, the synthesis of RNA was decreased concomitantly with that of DNA. Of course, it is possible that the fall in uridine incorporation into RNA was largely secondary to the inhibition of DNA synthesis.

In human lymphocytes both difluoromethylornithine and methylglyoxal bis(guanylhydrazone) quite effectively prevented the accumulation of spermidine and spermine in response to the lectin stimulation. The synthetic rates of DNA and protein

were profoundly (85–90%) inhibited by the combination of drugs, which clearly shows the absolute importance of polyamines for cell growth. The fact that the observed inhibition of synthesis of nucleic acids and protein in the presence of both drugs was mediated through intracellular polyamine depletion rather than being due to toxic effects was supported by several lines of evidence. The rate of glycolysis and glucose oxidation in stimulated lymphocytes remained practically unchanged despite the marked decrease in polyamine accumulation 24h after lymphocyte stimulation. There was good correlation between the difluoromethylornithine-induced decrease in protein synthesis and the decrease in the concentration of spermidine. Furthermore, the deleterious effects of polyamine inhibitors on macromolecular synthesis could be fully restored by low concentrations of exogenous polyamines. It is also worth noting that the resumption of the synthesis of DNA, RNA and protein occurred in the presence of polyamine concentrations not exceeding those normally found in activated lymphocytes. However, the reversibility of the effects exerted by methylglyoxal bis(guanylhydrazone) by spermidine and spermine could be, at least partially, based on competition of the polyamines for a common uptake system (Dave & Caballes, 1973). It is unlikely that a similar phenomenon could also occur with difluoromethylornithine.

In the presence of methylglyoxal bis(guanylhydrazone) the accumulation of spermidine and spermine was largely prevented, whereas the accumulation of putrescine was enhanced. This may imply that putrescine cannot take over the functions of spermidine and spermine. Depletion of spermidine (with no change in spermine) by difluoromethylornithine and methylglyoxal bis(guanylhydrazone) 24h after lectin stimulation was accompanied by a decrease in protein synthesis (Table 6). This finding supports the idea of an important role for spermidine in cell growth (Algranati & Goldemberg, 1977; Mamont *et al.*, 1978). A comparison of the effectiveness of different polyamines in restoring macromolecular synthesis after the inhibitor treatments was complicated by the interconversions of the added polyamines. However, it appeared that exogenous spermine alone was sufficient for the restoration of cell growth (Tables 1 and 4).

These results are in general agreemenent with the data of Krokan & Eriksen (1977), who reported that the inhibition of thymidine incorporation into DNA in HeLa cells was accompanied by parallel changes in the synthesis of RNA and protein. Furthermore, the present data are in a very good accordance with those obtained from studies on polyamine-deficient mutants of *Escherichia coli*. When these mutants were supplemented with exogenous polyamines, the cellular protein synthesis was stimulated before RNA and DNA synthesis (Young & Srinivasan, 1972). The impairment of protein synthesis in these mutants appears to be due to defects in the biosynthesis and/or assembly of small ribosomal subunits (Algranati & Goldemberg, 1977). Even though inhibition of general protein synthesis may be reflected in the observed disturbances in the synthesis of nucleic acids, independent and direct action of polyamines on the metabolism of nucleic acids was not, by any means, ruled out in our study. It is even likely that polyamines, as organic cations whose concentrations can be precisely regulated, take part in several metabolic processes and structural functions in the cell, and an absolute structural specificity of the polyamines is possibly only required in a few specific cases.

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