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Response of enzymes involved in the metabolism of polyamines to phytohaemagglutinin-induced activation of human lymphocytes

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The stimulation of lymphocyte ornithine decarboxylase and adenosylmethionine decarboxylase produced by phytohaemagglutinin was accompanied by an equally marked, but delayed, stimulation of spermidine synthase, which is not commonly considered as an inducible enzyme. In contrast with the marked stimulation of these biosynthetic enzymes, less marked changes were observed in the biodegradative enzymes of polyamines in response to phytohaemagglutinin. Diamine oxidase activity was undetectable during all stages of the transformation. The activity of polyamine oxidase remained either constant or was slightly decreased several days after addition of the mitogen. The activity of polyamine acetylase (employing all the natural polyamines as substrates) distinctly increased both in the cytosolic and crude nuclear preparations of the cells during later stages of mitogen activation. Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, although powerfully inhibiting ornithine decarboxylase, produced a gradual enhancement of adenosylmethionine decarboxylase activity during lymphocyte activation, without influencing the activities of the two propylamine transferases (spermidine synthase and spermine synthase).

Mitogen-activated mammalian lymphocytes have offered a useful model for studies devoted to the metabolism of polyamines during the transition of animal cells from the non-dividing state to the dividing state. Not only is the activation of lymphocytes accompanied by large increases in the biosynthesis of polyamines (Kay & Cooke, 1971; Kay & Lindsay, 1973; Fillingame & Morris, 1973*a*,*b*), but an enhanced accumulation of polyamines appears to be a prerequisite for cell division to occur, since lymphocyte proliferation can be largely prevented by inhibitors of adenosylmethionine decarboxylase (Otani *et al.*, 1974; Fillingame *et al.*, 1975) and ornithine decarboxylase (Hölttä *et al.*, 1979).

The marked stimulation of the biosynthetic decarboxylases of the polyamines in response to mitogens is well documented (Kay & Cooke, 1971; Kay & Lindsay, 1973; Fillingame & Morris, 1973*b*), but the behaviour of the two propylamine transferases during lymphocyte transformation has not been studied. The possible contribution, if any, of the biodegradative enzymes to the intracellular concentrations of the polyamines is likewise largely unknown. It is known, however, that putrescine and spermidine undergo monoacetylation in cultured human lymphocytes (Menashe *et al.*, 1980).

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Experimental

Cell cultures

Human peripheral lymphocytes were purified from the buffy-coat fraction, using a modification (Hovi *et al.*, 1976) of the method of Böyum (1968). The lymphocyte preparations were further purified by removal of most of the monocytes, which were allowed to attach to plastic dishes during overnight incubation in the growth medium. The cells (2×10^6 lymphocytes/ml) were cultured as previously described (Hölttä *et al.*, 1979).

Chemicals

Purified phytohaemagglutinin (type HA 16) was obtained from Wellcome Research Laboratories (Beckenham, Kent, U.K.). DL-[1-^{14}C]Ornithine (sp. radioactivity 58 Ci/mol), [^{14}C]acetyl-CoA (sp. radioactivity 56.6 Ci/mol), [^{14}C]putrescine (sp. radioactivity 116 Ci/mol) and [^{14}C]spermine (sp.

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radioactivity 122 Ci/mol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). *S*-Adenosyl-L-[1-^{14}C]methionine was prepared enzymically by the method of Pegg & Williams-Ashman (1969). Radioactive decarboxylated *S*-adenosylmethionine (labelled at C-2 of the original methionine moiety) was prepared and purified by the method of Pösö *et al.* (1976). DL-α-Difluoromethylornithine was a generous gift from the Centre de Recherche Merrell International (Strasbourg, France).

Preparation of cell fractions and extracts

For the acetylase assays lymphocytes (4×10^7 cells) were suspended in 1 ml of 50 mM-Tris/HCl buffer (pH 7.5) containing 0.25 M-sucrose, 25 mM-KCl and 5 mM-$MgCl_2$ and homogenized with 20 strokes in a Dounce homogenizer. Crude nuclei were pelleted by centrifugation at 800 *g* for 5 min. The nuclei were washed once and resuspended in the above buffer. The cytosolic fraction was obtained by centrifugation at 100000 *g* for 30 min.

For the other enzyme assays the lymphocytes (2×10^7 cells) were suspended in 1.5 ml of 25 mM-Tris/HCl buffer (pH 7.1) containing 0.1 mM-EDTA and 1 mM-dithiothreitol. The cells were then disintegrated by sonication with a Branson sonifier (3×5 s). The cell lysate was centrifuged at 100000 *g* for 30 min, and the resulting supernatant fraction was used as a source of enzyme.

The experiments were always performed in duplicate.

Analytical methods

The activity of ornithine decarboxylase was assayed principally as described by Jänne & Williams-Ashman (1971*a*). The assay mixture (0.25 ml) consisted of 50 mM-Tris/HCl buffer (pH 7.1), 1.2 μCi of DL-[1-^{14}C]ornithine, 0.05 mM-L-ornithine, 0.2 mM-pyridoxal phosphate, 4 mM-dithiothreitol, 4 mM-EDTA and enzyme protein. Adenosylmethionine decarboxylase activity was determined in the presence of 2.5 mM-putrescine with 0.2 mM-*S*-adenosyl-L-[1-^{14}C]methionine (sp. radioactivity 10 Ci/mol) as the substrate (Jänne & Williams-Ashman, 1971*b*).

The assays of spermidine synthase and spermine synthase were carried out with 0.03 mM-[^{14}C]decarboxylated *S*-adenosylmethionine (sp. radioactivity 7.4 Ci/mol) and 1 mM-putrescine/spermidine as the substrates respectively as described by Raina *et al.* (1976). Spermine synthase activity was also measured using a slightly modified method (Pajula *et al.*, 1979).

Diamine oxidase activity was determined by the method of Okuyama & Kobayashi (1961), as modified by Tryding & Willert (1968) with 0.05 mM-[^{14}C]putrescine (1 μCi/ml) as the substrate. The activity of polyamine oxidase was measured at the substrate concentration of 0.08 mM-[^{14}C]-spermine (2 μCi/ml) as described previously (Hölttä, 1977) and that of polyamine acetylase by the method of Libby (1978) as modified by Matsui & Pegg (1980). The acetylase assays were performed usually at 37°C (instead of 30°C). The reaction was linear with respect to the amount of enzyme (<0.1 mg) and reaction times (15–30 min).

Polyamines were determined after dansylation by the method of Seiler (1970) as modified by Dreyfuss *et al.* (1973) with aluminium oxide thin-layer plates in chloroform/dioxan/butan-1-ol (48:1:1, by vol.) for the separation of the dansylated amines. Protein was measured by the method of Lowry *et al.* (1951).

Results

Phytohaemagglutinin-induced changes of the biosynthetic enzymes of the polyamines

Quiescent human peripheral blood lymphocytes (cultured for 24 h in the RPMI 1640 medium) contained very low ornithine decarboxylase and adenosylmethionine decarboxylase activities. The specific activities of the two propylamine transferases (spermidine synthase and spermine synthase) were markedly higher than those of the two decarboxylases.

The addition of phytohaemagglutinin produced a marked sequential stimulation of the whole biosynthetic pathway of the polyamines. The marked stimulation of ornithine decarboxylase and adenosylmethionine decarboxylase activities was accompanied by an equally impressive, yet somewhat later, enhancement of spermidine synthase activity. Spermidine synthase reached its maximum around day 3, when the activities of the two decarboxylases were already decreased to the pretreatment values. The activity of spermine synthase likewise showed a moderate increase 3–4 days after the addition of phytohaemagglutinin (Table 1).

The pattern of polyamine accumulation during lymphocyte activation was similar to that published previously (Hölttä *et al.*, 1979). The cellular content of spermidine co-increased with the activity of spermidine synthase. The later accumulation of spermine occurred at the time of the increase in DNA accumulation (results not shown).

Effect of polyamines on the induction of ornithine decarboxylase and adenosylmethionine decarboxylase in phytohaemagglutinin-stimulated lymphocytes

The stimulation of ornithine decarboxylase by phytohaemagglutinin was inhibited in the presence of micromolar concentrations of putrescine, spermidine (Kay & Lindsay, 1973) and spermine

Table 1. *Effect of difluoromethylornithine on the activities of the polyamine-synthesizing enzymes*
Lymphocytes were incubated in the absence or presence of phytohaemagglutinin (PHA; 1 μg/ml) and difluoromethylornithine (DFMO; 2 mM) for the indicated times. Spermidine and spermine (10 μM) were added to the indicated cultures at 48 h. For the determination of the enzyme activities see the text.

Time (h)	Additions	Activity (pmol/mg of protein per h) Ornithine decarboxylase	Adenosylmethionine decarboxylase	Spermidine synthase	Spermine synthase
24	Control	20	70	2000	<2000
24	PHA	5090	1820	4940	<2000
24	PHA + DFMO	170	7650	6570	<2000
48	Control	<20	420	<2000	<2000
48	PHA	1060	2990	27300	3630
48	PHA + DFMO	130	36400	23600	2620
72	Control	<20	640	<2000	2000
72	PHA	38	830	25800	6220
72	PHA + DFMO	46	42800	35200	3400
72	PHA + DFMO + spermidine	25	6220	28300	5050
72	PHA + DFMO + spermine	25	1410	24700	4160

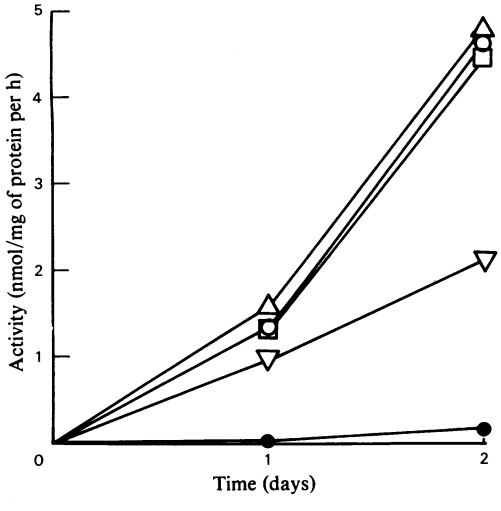

Fig. 1. *Effect of polyamines on the induction of adenosylmethionine decarboxylase in phytohaemagglutinin-activated lymphocytes*
Lymphocytes were incubated in the absence (●) or presence of phytohaemagglutinin alone (○) or together with 5 μM-putrescine (□), spermidine (△) or spermine (▽) for 24 h and 48 h. The enzyme activities are expressed as the means of duplicate assays from two parallel cultures.

(results not shown). Fig. 1 shows that the stimulation of adenosylmethionine decarboxylase remained unaffected by putrescine and spermidine (at concentrations inhibitory to ornithine decarboxylase) as in 3T3 cells (Clark & Fuller, 1975), but was inhibited by spermine. None of the polyamines exerted any effect on the stimulation of spermidine synthase by the mitogen (results not shown).

Effect of phytohaemagglutinin on the oxidation and acetylation of polyamines in lymphocytes

In contrast with diamine oxidase, the activity of which was undetectable in both resting and activated human lymphocytes, the cells appeared to contain rather high (in comparison with the biosynthetic enzymes) polyamine oxidase activity. Polyamine oxidase activity remained relatively constant during the first days of lymphocyte transformation, whereafter a slight decrease (up to 50% during extensive lymphocyte stimulation) in the enzyme activity was observed (Fig. 2). However, if the proliferation response of the cells to phytohaemagglutinin was only moderate, the activity of polyamine oxidase remained unchanged.

The contribution of polyamine oxidase activity to the regulation of cellular concentrations of putrescine, spermidine and spermine thus appears to be minimal. Whether the acetylation of polyamines (Matsui & Pegg, 1980) could be the rate-controlling step of polyamine degradation by polyamine oxidase remains to be seen. In our study we found that during later stages of lymphocyte transformation the capacity for the acetylation of all polyamines was clearly enhanced in the cytosolic cell preparations (Table 2). The acetylation rate of spermidine was higher than that of putrescine and spermine (Table 2). The patterns of the acetylase activities in the crude nuclear preparations were fairly similar to those in the cytosolic fractions, although the increases in the enzyme activity at 72 h after stimulation were somewhat smaller (results not shown).

Table 2. *Cytosolic polyamine acetylase activities during lymphocyte transformation*
Lymphocytes were incubated in the absence or presence of phytohaemagglutinin (PHA; 1 μg/ml) for the indicated times, after which the cytosolic enzyme activities were determined as described in the Experimental section. The values are means for duplicate determinations.

Time (h)	Additions	Substrate ...	Acetylase activity (pmol/mg of protein per 30 min) Putrescine	Spermidine	Spermine
24	Control		105	430	95
24	PHA		165	620	115
48	Control		75	360	50
48	PHA		97	450	92
72	Control		60	420	50
72	PHA		105	1750	270

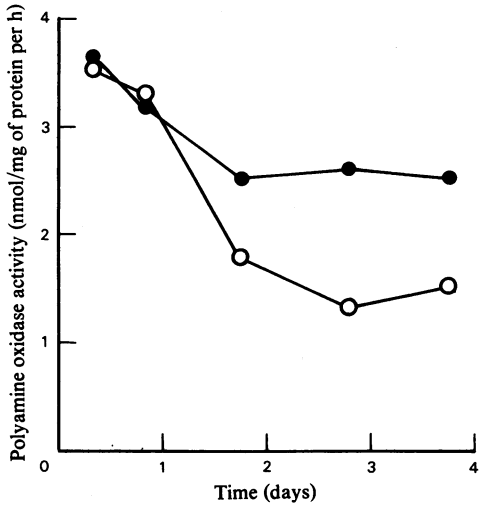

Fig. 2. *Polyamine oxidase in control and phytohaemagglutinin-induced lymphocytes*
Lymphocytes were incubated in the absence (●) or presence (○) of phytohaemagglutinin for the times indicated and assayed for the polyamine oxidase activity as described in the Experimental section.

Effect of difluoromethylornithine on the biosynthetic enzymes of polyamines during lymphocyte activation

Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978), although effectively inhibiting ornithine decarboxylase activity and rapidly decreasing the concentrations of putrescine and spermidine, leads to a paradoxical stimulation of adenosylmethionine decarboxylase in several experimental systems (Mamont *et al.*, 1978; Prakash *et al.*, 1978; Alhonen-Hongisto, 1980). Even though the molecular mechanism of this stimulation remains to be determined, the enhanced enzyme activity is rapidly repressed in the presence of low concentrations of spermidine or spermine (Mamont *et al.*, 1978; Alhonen-Hongisto, 1980). As shown in Table 1, the inclusion of difluoromethylornithine (2 mM) together with phytohaemagglutinin abolished any stimulation of ornithine decarboxylase, but produced at the same time a remarkable increase in the activity of adenosylmethionine decarboxylase. The stimulation of the latter enzyme was effectively prevented by low concentrations of spermidine or spermine (Table 1). Even though the mitogen produced a similar increase, as regards the magnitude, in spermidine synthase activity, no further enhancement of the enzyme activity was brought about by the addition of difluoromethylornithine. Spermine synthase activity was likewise largely unaffected by difluoromethylornithine in phytohaemagglutinin-activated lymphocyte cultures (Table 1).

Discussion

Our results clearly show that the transition of quiescent peripheral lymphocytes to the dividing stage in response to phytohaemagglutinin not only involves a marked stimulation of ornithine decarboxylase and adenosylmethionine decarboxylase activities (Kay & Cooke, 1971; Kay & Lindsay, 1973), but the whole biosynthetic pathway of polyamines is powerfully activated. Spermidine synthase, a stable enzyme (for references see Raina & Jänne, 1975), has all the characteristics of a constitutive enzyme and there are few reports of its induction in contrast with the two biosynthetic decarboxylases of the polyamines. However, in some rodent tissues the activity of spermidine synthase may markedly increase in response to tissue loss (Hannonen *et al.*, 1972) or to various hormones (Oka *et al.*, 1977; Käpyaho *et al.*, 1980). During the activation of peripheral human blood lymphocytes, the stimulation of spermidine synthase was similar to that of ornithine decarboxylase and adenosylmethionine decarboxylase. The swift

accumulation of spermidine in activated human lymphocytes (Hölttä *et al.*, 1979) may thus be related to the marked stimulation of spermidine synthase activity observed in the present study.

Several lines of evidence suggested that, besides the sequential induction of the two decarboxylases and spermidine synthase, there is no common co-ordinated regulatory mechanism for the three enzymes. The stimulation of ornithine decarboxylase is effectively prevented by low concentrations of all the natural polyamines, not only in activated human lymphocytes, but also in several other systems (for references see Jänne *et al.*, 1978). The phytohaemagglutinin-induced stimulation of adenosylmethionine decarboxylase could be partially inhibited only by spermine, putrescine and spermidine being ineffective, whereas the induction of spermidine synthase was not influenced by any of the polyamines.

Polyamine depletion produced by inhibitors of ornithine decarboxylase (Mamont *et al.*, 1978; Alhonen-Hongisto, 1980; Table 1) appears to trigger another regulatory mechanism leading to a swift enhancement of adenosylmethionine decarboxylase activity. The latter stimulation is effectively repressed by spermidine and/or spermine (Mamont *et al.*, 1978; Alhonen-Hongisto, 1980; Sakai *et al.*, 1980; Table 1). There is no evidence that spermidine synthase would be subject to similar control; at least, putrescine and spermidine depletion produced by difluoromethylornithine did not enhance spermidine synthase activity in activated lymphocytes (Table 1).

Although marked changes in polyamine biosynthesis have been reported in many experimental systems, studies on the biodegradation of polyamines are fragmentary and the whole area of research seems to be more or less neglected. However, recently pieces of experimental evidence have been accumulated to the extent that it is possible to outline the most probable degradative routes of polyamines in animal tissues. Owing to the limited tissue and/or species distribution of both diamine oxidase and plasma amine oxidase (for references see Kapeller-Adler, 1970), the contribution of these enzyme activities to the tissue concentrations of polyamines (like human lymphocytes in the present study) are in all likelihood of minor importance, with the possible exception of some specific tissues (Jänne & Hölttä, 1973; Baylin *et al.*, 1978). Similarly, the discovery of the peroxisomal polyamine oxidase (Hölttä, 1977), capable of cleaving spermine to spermidine and spermidine to putrescine, has not in itself clarified the degradation routes of polyamines, since the activity of this enzyme only shows minor changes under conditions of enhanced polyamine interconversions *in vivo* (Hölttä *et al.*, 1973). However, two important findings have aided our understanding of the physiological degradation of polyamines in animal cells. Seiler & Al-Therib (1974) reported that putrescine can serve as a substrate for monoamine oxidase, which is universally distributed in animal cells, provided that the diamine is first monoacetylated. Matsui & Pegg (1980) observed that in rat liver after carbon tetrachloride treatment, at the time of enhanced interconversions of the polyamines, the acetylation of spermidine and spermine was markedly increased. These findings, together with the fact that acetylated polyamines may serve as substrates for polyamine oxidase (Hölttä, 1977; Blankenship, 1979), strongly suggest that acetylated polyamines could act as the natural substrates for polyamine oxidase *in vivo*.

It is likewise clear from the present results that the activity of polyamine oxidase only showed minor changes during lymphocyte activation, whereas the acetylation of all polyamines was substantially increased at later stages of lymphocyte transformation. The acetylation of polyamines may thus be a means for a physiological inactivation of these compounds through subsequent oxidation by polyamine oxidase, via enhanced excretion from the cells or by virtue of their altered cation properties.

In conclusion, the cellular concentrations of polyamines during lymphocyte transformation are mainly regulated by the activities of the biosynthetic enzymes of polyamines, and the degradative enzymes appear to be of minor regulatory importance.

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