The effect of time of addition of glutamine or nucleosides on proliferation of rat cervical lymph-node T-lymphocytes after stimulation by concanavalin A

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1. Addition of concanavalin A to T-cell lymphocytes from rat cervical lymph nodes increases the activity of glutaminase within 1 h and those of carbamoyl-phosphate synthase II and aspartate transcarbamoylase within 3 h. There was a similar time course for the effects of concanavalin A on rates of glutamine utilization, which was increased within 1 h, and on pyrimidine nucleotide synthesis, which was increased by 40 % at 2 h and by 100 % at 3 h. 2. A delay in the addition of glutamine to the culture medium after addition of concanavalin A caused a decrease in [³H]thymidine incorporation only after 4–6 h. In the absence of glutamine, delay in addition of guanosine or inosine caused a decrease in [³H]thymidine incorporation only after 6–8 h after the addition of concanavalin A. 3. In contrast, a delay in addition of adenosine or uridine to the culture medium had an immediate effect (i.e. within 2 h) on the rate of incorporation of [³H]thymidine. It is suggested that adenosine and uridine have specific effects on proliferation via specific receptors for these nucleosides in the membrane.

INTRODUCTION

It has been shown that the rate of glutamine utilization by isolated incubated mesenteric lymphocytes is very high and that the oxidation is only partial (Ardawi & Newsholme, 1983, 1985). The significance of this high rate of glutaminolysis has been considered previously for rapidly dividing cells, including tumour cells (see Krebs, 1980; McKeehan, 1982; Kovacevic & McGivan, 1983). Two suggestions have been made; first, the pathway provides a considerable amount of ATP, so that the importance of glutaminolysis might be primarily for energy formation in these cells; second, the pathway provides intermediates for the biosynthesis of purine and pyrimidine nucleotides, which are essential for the cell cycle. However, there are difficulties with both these explanations. First, if energy generation itself were important, it might be expected that more of the carbon of the glutamine would be fully oxidized, via the complete tricarboxylic acid cycle, which appears to be present in these cells (Ardawi & Newsholme, 1985). Second, the rate of the synthesis and even the maximum capacity for synthesis of pyrimidine nucleotides by lymphocytes has been shown to be very small in comparison with the rate at which glutamine is utilized by these cells (Szondy & Newsholme, 1989).

A new hypothesis has been proposed which suggests that a high rate of glutaminolysis is important to provide optimal conditions for the precise regulation of the rates of synthesis of purine and pyrimidine nucleotides at specific times during the cell cycle: it is considered that glutaminolysis provides a dynamic buffer for intermediates such as glutamine and aspartate, which are precursors for purine and pyrimidine nucleotide synthesis (Newsholme *et al.*, 1985a,b). Consistent with this hypothesis, it has recently been shown that, for proliferation of lymph-node lymphocytes, glutamine can be partially replaced by addition of nucleosides to the incubation medium (Szondy & Newsholme, 1990).

To provide further information on the role of glutaminolysis for proliferation, at time course of the effects of provision of

glutamine or nucleosides on the rate of [³H]thymidine incorporation has been carried out with T-lymphocytes prepared from cervical lymph nodes. It is predicted that, if the major role of glutaminolysis is to provide optimal sensitivity for nucleotide synthesis, a similar time course for dependency upon glutamine and nucleosides for proliferation should be observed. Further, the rate of glutamine utilization, the activity of phosphate-dependent glutaminase (EC 3.5.1.2) (the rate-limiting enzyme in glutaminolysis) and that of carbamoyl-phosphate synthase II (EC 6.3.5.5) (the rate-limiting enzyme in pyrimidine synthesis) and the rate of incorporation of H¹⁴CO₃⁻ into pyrimidine nucleotides has been measured in T-lymphocytes at various time points after concanavalin A stimulation.

MATERIALS AND METHODS

Animals, chemicals and enzymes were obtained from sources given previously (Szondy & Newsholme, 1989, 1990.) [2-¹⁴C]Carbamoyl phosphate (13.3 mCi/mmol; Li⁺ salt) was obtained from New England Nuclear, Stevenage, Herts., U.K.

Preparation of T-lymphocytes

Lymphocytes were prepared from cervical lymph nodes as described previously (Ardawi & Newsholme, 1983). T-lymphocytes were purified as described by Hunt (1987): in brief, T-lymphocytes were separated from B-lymphocytes by a method based on the presence of anti-Ig antibodies on the cell surface of B-, but not T-, lymphocytes. Anti-Ig-antibody-coated sheep erythrocytes were mixed with lymphocytes prepared from the lymph nodes. After incubation for 20 min, 3 ml of the mixture was layered on the top of 4 ml of Histopaque (Sigma) and centrifuged at 2500 *g* for 20 min. The proportion of T-cells remaining in the supernatant was 75–80 %.

The cells for the short-term incubations were used immediately, whereas for the proliferation experiments they were kept overnight in the absence of glutamine in order to deplete their

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intracellular glutamine concentration. Concanavalin A (5 $\mu\text{g/ml}$) was added at zero time: glutamine (final concn. 1 mM) or nucleosides (final concn. 100 μM) was added to the culture at zero time or at each second hour after zero time. [^3H]Thymidine was also added at zero time, and the incorporation was determined at 24 h after zero time.

Cell culture

Lymphocytes were cultured in wells of micro-titre culture plates (approx. 5×10^5 cells in 100 μl of medium were present in each 200 μl well) in a Hepes-buffered RPMI medium containing 10% (v/v) foetal-calf serum, 100 units of streptomycin/ml and 200 units of penicillin/ml [the foetal-calf serum was dialysed against phosphate-buffered saline (Cuvencor & Weidemann, 1976) in order to remove glutamine and other small molecules.] The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in air. [^3H]Thymidine (0.2 μCi) was added to each 200 μl well at zero time of the culture, and the proliferation response was measured from the incorporation of [^3H]thymidine over a period of 24 h. Cultures were harvested on to glass-fibre filters with an automated cell harvester. The filters were washed and dried: the radioactivity on the dried filters was measured in a liquid-scintillation counter (Beckman LS 7500). Evidence that such incorporation is a measure of proliferation has been given elsewhere (Szondy & Newsholme, 1990).

Incubation procedure

Incubations were performed at 37 °C in 10 ml silicone-treated Erlenmeyer flasks in a total volume of 1.0 ml of phosphate-buffered saline (Ardawi & Newsholme, 1983) which had been oxygenated with 100% O_2 for 30 min, and which was supplemented with 5% (w/v) defatted BSA and 5 mM-glucose at pH 7.4. The lymphocytes were incubated in the absence of presence of 5 μg of concanavalin A/ml, and substrates for pyrimidine nucleotide synthesis or glutaminolysis were added at 0, 1 and 2 h after concanavalin A activation. The incubation medium contained 2 mM-glutamine when glutamine utilization was to be measured, or 2 mM-glutamine, 15 mM- KHCO_3 and 40 μCi of $\text{NaH}^{14}\text{CO}_3$ when the rate of pyrimidine nucleotide synthesis was to be measured. Each incubation contained 5×10^7 cells; the gas phase was 100% O_2 . The reaction was stopped by addition of 200 μl of ice-cold 25% (w/v) HClO_4 60 min after substrate addition. The results are expressed as percentages of the non-stimulated control.

Determination of rates of glutamine utilization and pyrimidine synthesis

The glutamine concentration in the incubation medium was measured by the method of Windmueller & Spaeth (1974). The rate of pyrimidine nucleotide synthesis was measured from the ^{14}C incorporation from $\text{H}^{14}\text{CO}_3^-$ into uridine nucleotides. Uridine nucleotides from the acid-soluble cell fraction were converted into UMP by heating to 100 °C for 10 min in acid, and it was isolated by crystallization (Crandall *et al.*, 1978). The radioactivity was measured in a liquid-scintillation counter (Beckman LS 7500). A high concentration of bicarbonate was used in these experiments (15 mM), to minimize any effects of a decrease in the bicarbonate concentration that might occur during the 3 h incubation period. The fact that in the control incubation the rate of pyrimidine synthesis, based on ^{14}C incorporation from bicarbonate, was linear with time for 3 h (Table 2) supports the view that any decrease in bicarbonate concentration was without significant effect.

Enzyme assays

Cells were collected at various times of incubation up to 3 h after concanavalin A addition, and were then resuspended in 500 μl of 150 mM-potassium phosphate, pH 7.4, containing 30% (w/v) dimethyl sulphoxide, 5% (w/v) glycerol and 1 mM-dithiothreitol, for measuring carbamoyl-phosphate synthase II and aspartate transcarbamoylase activity, and in 150 mM-potassium phosphate buffer (equimolar mixture of K_2HPO_4 and KH_2PO_4 , 1 mM-EDTA and 50 mM-Tris/HCl, pH 8.6), for measuring phosphate-dependent glutaminase activity. Cells were sonicated and the crude homogenate was used to measure the glutaminase activity as described by Ardawi & Newsholme (1984) or centrifuged at 100 000 g for 10 min and the cytosol fraction was used for measuring the carbamoyl-phosphate synthase and aspartate transcarbamoylase activities as described by Aoki *et al.* (1982). Protein was determined by the method of Bradford (1976) with globulin as a standard.

RESULTS AND DISCUSSION

Incubation of lymphocytes for 3 h in the absence of concanavalin A leads to a small but non-significant decrease in glutaminase activity, whereas addition of concanavalin A increases the activity almost 4-fold by 3 h (Table 1). As shown previously (Ardawi & Newsholme, 1983), addition of the mitogen concanavalin A increases the rate of glutamine utilization; after 3 h incubation the increase is approx. 5-fold. This change is similar to that of the enzyme activity, which supports the view the activity of glutaminase is important in the control of the rate of glutamine utilization in these cells.

Addition of concanavalin A also results in an increase in the rate of pyrimidine synthesis, although this increase occurs 60 min later than the increase in the rate of glutaminolysis: the increase in rate is 6, 41 and 96% after 60, 120 and 180 min respectively (Table 2). The magnitude of the increase in carbamoyl phosphate synthase activity is similar to that of the rate of pyrimidine nucleotide synthesis, and they both show a similar time course; this finding supports the view that the activity of carbamoyl-phosphate synthase is important in the control of the rate of pyrimidine nucleotide synthesis. This enzyme is part of a multi-functional protein that contains aspartate transcarbamoylase and dihydro-orotase, the second and the third enzymes of the pyrimidine-nucleotide-synthesis pathway. The fact that carbamoyl-phosphate synthase and aspartate transcarbamoylase activities increased simultaneously (Table 1) suggests that the increase in enzyme activity is a result of an increase in the amount of the enzyme protein.

The effects of addition of glutamine or nucleosides, at different times after addition of concanavalin A, on [^3H]thymidine incorporation are shown in Table 3. Under the conditions of this experiment, the effect of addition of glutamine (1 mM) to the culture medium was to increase the rate of [^3H]thymidine incorporation by approx. 6-fold. There was no effect on incorporation of a delay in addition of glutamine for 2 or 4 h after zero time. However, incorporation was decreased when glutamine addition occurred at 6 h after zero time. Hence the effect of a delay in addition of glutamine occurred between 4 and 6 h after zero time. The 'reverse experiment' produced a similar time course; glutamine was added at zero time, but was then removed by addition of asparaginase at different time points: incorporation was very low (similar to that in the absence of added glutamine) when asparaginase was added at 0, 2 and 4 h; but it was doubled when glutamine removal was delayed until 6 h after zero time (results not shown). These findings suggest that, although glutamine utilization is increased within 1 h after concanavalin A addition (see Table 1), it is not required to

Table 1. Effect of concanavalin A (Con A) on the maximum activities of glutaminase, carbamoyl-phosphate synthase II (CPS-II) and aspartate transcarbamoylase in T-cell lymphocytes during the first 3 h of culture

Results are presented as means $\pm$ S.E.M. for four separate experiments. Statistical significance from controls (Student's *t* test) is indicated by * ($P < 0.05$).

Time after addition of Con A (min)	Enzyme activities (nmol/h per mg of protein)					
	Glutaminase		CPS-II		Aspartate transcarbamoylase	
	Control	Con A	Control	Con A	Control	Con A
0	2106 $\pm$ 262	—	3.96 $\pm$ 0.56	—	122.5 $\pm$ 21.1	—
60	2050 $\pm$ 316	3669 $\pm$ 270*	—	—	—	—
90	1990 $\pm$ 450	4955 $\pm$ 637	3.67 $\pm$ 0.21	4.60 $\pm$ 0.53	134.2 $\pm$ 25.7	179.8 $\pm$ 16.3
150	1887 $\pm$ 367	8690 $\pm$ 591*	—	—	—	—
180	1791 $\pm$ 471	13 706 $\pm$ 685*	3.48 $\pm$ 0.11	9.15 $\pm$ 0.47*	125.3 $\pm$ 12.6	338 $\pm$ 27.6

Table 2. Effects of concanavalin (Con A) on rates of glutamine utilization and pyrimidine nucleotide synthesis in T-cell lymphocytes during 180 min of culture

Results are presented as means $\pm$ S.E.M. for four separate experiments. Statistical significance (Student's *t* test) is indicated by * ($P < 0.05$).

Time after addition of Con A (min)	Rates (nmol† or pmol‡/h per mg of protein)			
	Glutamine utilization†		Pyrimidine synthesis‡	
	Control	Con A	Control	Con A
60	83.9 $\pm$ 6.1	125.8 $\pm$ 12.1*	81.4 $\pm$ 2.6	86.6 $\pm$ 2.4
120	80.4 $\pm$ 5.7	249.1 $\pm$ 17.0*	80.2 $\pm$ 4.1	113.8 $\pm$ 6.1*
180	78.6 $\pm$ 4.1	467.5 $\pm$ 21.7*	76.5 $\pm$ 5.2	149.9 $\pm$ 11.7*

Table 3. Effect of time of addition of glutamine or nucleosides after addition of concanavalin A on [³H]thymidine incorporation in cervical T-lymphocytes of the rat

At zero time, concanavalin (Con A) and [³H]thymidine were added to the culture medium: glutamine (1 mM) or nucleoside (100 μ M) was added at zero time, 2, 4, 6, 8 or 10 h later. Cultures were harvested 24 h after addition of [³H]thymidine. Results are presented as means $\pm$ S.E.M. for four separate experiments: a statistically significant difference from the zero-time incorporation is indicated by * ($P < 0.05$) or ** ($P < 0.01$). The incorporation for 24 h in the absence of glutamine or nucleoside was 98 $\pm$ 2 c.p.m.

Time of addition of glutamine or nucleoside after addition of Con A (h)	Incorporation of [³ H]thymidine (c.p.m.)					
	Glutamine (1 mM)	Adenosine (0.1 mM)	Guanosine (0.1 mM)	Uridine (0.1 mM)	Inosine (0.1 mM)	Cytosine (0.1 mM)
0	650 $\pm$ 167	399 $\pm$ 22	326 $\pm$ 14	476 $\pm$ 17	310 $\pm$ 11	94 $\pm$ 22
2	604 $\pm$ 153	292 $\pm$ 38*	311 $\pm$ 27	191 $\pm$ 8**	300 $\pm$ 17	87 $\pm$ 16
4	380 $\pm$ 128	298 $\pm$ 52*	305 $\pm$ 18	227 $\pm$ 73**	304 $\pm$ 11	81 $\pm$ 11
6	324 $\pm$ 84*	304 $\pm$ 39*	291 $\pm$ 28	206 $\pm$ 26*	290 $\pm$ 20	80 $\pm$ 26
8	326 $\pm$ 38*	249 $\pm$ 24**	221 $\pm$ 14**	224 $\pm$ 24**	205 $\pm$ 17**	88 $\pm$ 17
10	250 $\pm$ 166*	263 $\pm$ 46**	180 $\pm$ 15**	163 $\pm$ 49**	160 $\pm$ 21**	81 $\pm$ 32

support the proliferative process until between 4 and 6 h after the initiation of the proliferative process. Furthermore, lack of glutamine at this time point appears to have an almost 'threshold' effect, since the effect of a delay in addition of glutamine for more than 6 h caused only a further small decrease in incorporation, and it was not statistically significant (Table 3).

These findings are consistent with the views either that the glutaminolytic pathway provides ATP for these cells or that it provides optimal conditions for control of the precise rates of biosynthesis (branched-point sensitivity; Newsholme *et al.*, 1985a,b). Some further support for its role in branched-point

sensitivity is obtained from the experiments with the nucleosides. When guanosine or inosine was added to the culture medium, instead of glutamine, no effect was observed if these nucleosides were omitted from the culture medium until 6 h after zero time (Table 3). In the absence of nucleosides, glutamine would be required to synthesize purine and pyrimidine nucleotides, and its requirement for proliferation occurs at about the same time as that for these two nucleosides. The facts that the time courses for glutamine or nucleoside requirements are similar and that the rate of glutaminolysis is more than 400-times the rate of pyrimidine nucleotide synthesis (see Table 2) provide further

support for the hypothesis that the high rate of glutamine utilization in these cells is to provide branched-point sensitivity in control. It is important to note that the glutaminolytic pathway provides not only glutamine but also aspartate for pyrimidine synthesis. Thus, although the K_m of carbamoyl-phosphate synthase II for glutamine is low ($16.5 \mu\text{M}$; Szondy & Newsholme, 1990), that of aspartate transcarbamoylase for aspartate is high (9.1 mM), so that branched-point sensitivity might be particularly important for 'buffering' the aspartate concentration in lymphocytes. Nonetheless, despite the low K_m , the concentration of glutamine might also become limiting for carbamoyl-phosphate synthase II, since the activity of glutaminase is markedly increased in stimulated lymphocytes: a dynamic 'buffer' for glutamine concentration might also be of importance to permit precise increased rates of purine synthesis.

Addition of cytidine was found to inhibit incorporation at all time points: this confirms our previous findings (Szondy & Newsholme, 1990), and is probably caused by lack of cytidine deaminase in these cells, so that the proportion of cytidine and uridine nucleotides becomes unbalanced.

Of interest, delay in addition of adenosine or uridine to the culture medium had an immediate effect, that is, an effect detectable after only 2 h from zero time. These results suggest that adenosine and uridine have an additional role to that of guanosine, inosine (or glutamine) in the proliferation process. Since the effect occurs so early, it is suggested that they may play a specific regulatory role in cell proliferation. This is likely to be achieved through specific cell-surface receptors for these nucleosides on T-lymphocytes of the rat. A similar suggestion, for an adenosine receptor, based on a totally different line of investigation, was raised in previous work (Szondy & Newsholme, 1990). If correct, this hypothesis predicts that these two nucleo-

tides could have immunostimulatory effects for T-lymphocytes, at least in the rat.

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