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The oxidation of leucine in tumour-bearing rats

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Rats bearing the Walker-256 carcinosarcoma showed significant changes in leucine metabolism compared with their non-tumour-bearing controls. After a single intravenous tracer dose of L-[1-^{14}C]leucine *in vivo*, $^{14}CO_2$ release by tumour-bearing rats was significantly elevated throughout the time course of administration. In addition, both the clearance and turnover rates of the tracer were significantly enhanced in these animals. Incubation of soleus muscles from control and tumour-bearing rats in the presence of L-[1-^{14}C]leucine revealed an enhanced oxidation of the amino acid in the tumour-bearing group. Tumour tissue slices were also able to oxidize the tracer at a similar rate to that found in soleus muscles from control animals.

INTRODUCTION

Malignant tumour burden is a pathological state associated with decreased food intake and a considerable demand by the rapidly growing tumour for glucose and amino acids. Despite the controversy about the underlying molecular mechanism of cancer cachexia, the growing tumour has a considerable demand for essential amino acids. According to Lazo (1985), a tumour can increase considerably the daily need for leucine in humans, the increase having a better correlation with the clinical deterioration of the patient (Ruddon, 1981) than the modification of carbohydrate and energy metabolism. As a result of the demand for leucine, there is an amino acid flux from muscle to the tumour associated with muscular wastage in the host (Lazo, 1985).

The aim of the present paper is to examine the influence of a rapidly growing tumour, the Walker-256 carcinosarcoma, on leucine metabolism in the host.

EXPERIMENTAL

Animals and tumour inoculation

Female Wistar rats were used in all experiments. The animals were kept in a light- (on from 08:00 to 20:00 h), temperature- (21–22 °C) and humidity- (70–80 %) controlled room. They remained housed in individual polypropylene-bottomed cages with wood shavings as absorbent material. They were fed on A04 rat chow pellets (Panlab, Barcelona, Spain) *ad libitum* except where stated.

A Walker-256 carcinosarcoma cell suspension [approx. 10^7 cells, suspended in Krebs–Henseleit (1932) saline] was injected subcutaneously on the left flank of the rats, the control animals being sham-injected with the same volume of Krebs–Henseleit saline under light diethyl ether anaesthesia. The Walker-256 carcinosarcoma is a rapidly growing tumour with a volume doubling time of 0.86 day (Herzfeld & Greengard, 1972); the amount implanted ensured that the tumour mass was 3–5 % of carcass weight at the time of the experiments. We are very grateful to Dr. D. H. Williamson, Metabolic Research Laboratory, Radcliffe Infirmary, Oxford, U.K., for a generous supply of the tumour. Food intake and body weight were measured daily after implantation of the tumour. Control rats were 'pair-fed' with the tumour-bearing matched partners, and experiments were thence carried out after a 24 h delay. All experiments were carried out between 10:00 and 11:00 h, 10 days after tumour implantation.

Biochemicals and radioactive compounds

All biochemicals were from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Boehringer (Mannheim, Germany). L-[1-^{14}C]Leucine (sp. radioactivity 54 mCi/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.).

$^{14}CO_2$ production *in vivo*

Tracer doses of L-[1-^{14}C]leucine (4 μCi in 0.5 ml of 0.9 % NaCl solution) were administered via one of the lateral tail veins under light diethyl ether anaesthesia. As rapidly as possible, the animal was then placed in a 5-litre glass desiccator connected to a wash bottle fitted with a sintered-glass tube and containing 20 ml of Lumasorb (Lumac, Landgraaf, The Netherlands) to absorb the CO_2. Air was drawn through the system by means of an electrical pump at about 2 litres/min. The contents of the wash bottle were changed every 30 min for 120 min, and 5 ml of the Lumasorb was added directly to scintillation fluid for measurement of radioactivity (Oller do Nascimiento & Williamson, 1986).

L-[1-^{14}C]Leucine turnover rate

Another group of rats was injected with the same tracer doses of L-[1-^{14}C]leucine as described above through one of the tail veins. Samples (0.2 ml) of blood were collected from the tip of the tail at 2, 4, 8, 12 and 16 min after injection, and 2 ml of 6 % (w/v) $HClO_4$ was added to each sample, followed by centrifugation at 4 °C at 3000 rev./min for 10 min to separate the precipitated phase from the water-soluble one. The clear supernatants were neutralized with 30 % (w/v) KOH to pH 5–6 and, after centrifugation under the same conditions as described above, to eliminate the $KClO_4$ precipitate, the radioactivity was determined in 0.1 ml samples.

The half-life ($t_{0.5}$) and distribution volume of the injected L-[1-^{14}C]leucine were calculated from the intercept at $t = 0$ on a semi-logarithmic plot of the blood radioactivity values (Shipley & Clark, 1972). The linearity of this kind of plot between the time interval considered had a correlation coefficient of 0.997. The blood clearance of the tracer was calculated, assuming mono-exponential kinetics, from the formula:

$$\text{Clearance (ml/min)} = \text{distribution volume (ml)} \times 0.693/t_{0.5}\ \text{(min)}$$

where the last term represents the fractional decrease in leucine concentration per min. Turnover rate represents the total quantity of leucine moving through the pool per unit time, assuming a single pool system (Shipley & Clark, 1972).

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Table 1. Turnover rates of L-[1-^{14}C]leucine in tumour-bearing rats

For full details, see the Experimental section. The results are mean values ± S.E.M. for five to six different animals. Values that are significantly different by Student's *t* test from control values are indicated by $*P < 0.05$ and $**P < 0.01$.

Parameter	Experimental group	
	Control	Tumour-bearing
Blood leucine concn. (mM)	0.106 ± 0.013	0.156 ± 0.009*
$t_{0.5}$ (min)	2.90 ± 0.28	2.08 ± 0.20*
Distribution volume (ml/100 g body wt.)	27.1 ± 2.27	30.0 ± 2.43
Clearance rate (ml/min per 100 g body wt.)	6.53 ± 0.30	10.5 ± 1.59*
Turnover rate (μmol/min per 100 g body wt.)	0.71 ± 0.12	1.59 ± 0.16**

Blood leucine concentrations were determined with an automatic amino acid analyser (Rank–Hilger) with *o*-phthalaldehyde as amino-group reagent.

Experiments *in vitro*

The dissection and isolation of the soleus muscles were carried out under pentobarbital anaesthesia (60 mg/kg) as previously described (Maizels *et al.*, 1977). The isolated muscle was fixed to a stainless-steel clip, to maintain the muscle under slight tension. Both whole soleus muscles and pieces (20–30 mg) of tumour tissue were incubated separately in specially designed flasks provided with a central well, in a total volume of 4 ml of Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4. The medium also contained L-[1-^{14}C]leucine (sp. radioactivity 0.2 μCi/μmol) and 5 mM-glucose, except where stated. Flasks were sealed with rubber stoppers and incubated for 90 min in a thermostatically controlled bath (37 °C) with a shaking device (80 cycles/min). At the end of the incubation, Hyamine hydroxide (0.2 ml) was added to the centre well and the reaction was stopped by addition of 60% $HClO_4$ (0.2 ml) to the reaction medium. The wells were counted for radioactivity, to assess the amount of the amino acid that was oxidized to $^{14}CO_2$ during the incubation time. The incorporation of the tracer into the tissue was measured after solubilization of the tissue fragments for 2 h with NCS at 50 °C and decoloration with H_2O_2 (Peng, 1977). The solubilized tissue content was then mixed with liquid-scintillation cocktail and its radioactivity measured in a β-radiation counter.

The ATP content of both the muscle and tumoural tissue was measured before and after the incubation (Trautschold *et al.*, 1985).

RESULTS AND DISCUSSION

Oxidation of L-[1-^{14}C]leucine to $^{14}CO_2$ *in vivo*

As shown in Fig. 1, the pattern of cumulative $^{14}CO_2$ production from the L-[1-^{14}C]leucine administered to the animals was similar in both groups; however, the tumour-bearing rats showed significantly higher rates of leucine oxidation, expressed as a percentage of the cumulative dose. The rates were also higher in the tumour-bearing group during the first 60 min when expressed as a percentage of the administered dose. The cumulative percentage recoveries of the injected label as $^{14}CO_2$ at 2 h after the administration of the tracer were 29.5 ± 3.66 and 16.2 ± 2.25 for tumour-bearing and control rats respectively. Similar data have been previously reported both in control rats (White & Brooks, 1981) and in rats bearing a Morris hepatoma (Paxton *et al.*, 1988).

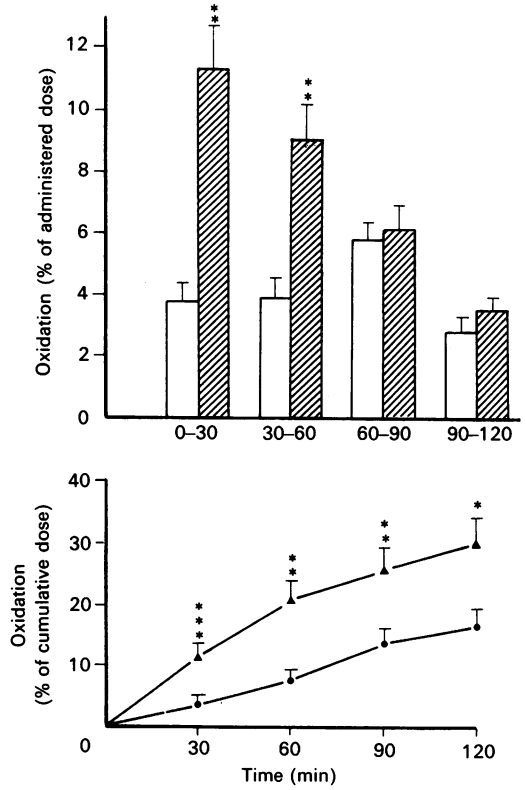

Fig. 1. Time course of the oxidation of [^{14}C]leucine to $^{14}CO_2$

For full details, see the Experimental section. The control group is indicated by □ and ●, and the tumour-bearing one by ▨ and ▲. [^{14}C]Leucine oxidation to $^{14}CO_2$ is expressed both as a percentage of the administered dose and as a percentage of the cumulative dose. The results are mean values ± S.E.M. The number of animals per group was five. Values that are significantly different by Student's *t* test from control values are indicated by $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

L-[1-^{14}C]Leucine distribution volume, $t_{0.5}$ and turnover rate

Bearing in mind the increased leucine oxidation in tumour-bearing rats described above, we decided to examine the blood disappearance kinetics of the injected leucine. As shown in Table 1, the presence of the tumour significantly decreased (by nearly 30%) the $t_{0.5}$ of the administered leucine. As a result of this change in $t_{0.5}$, and although no changes in distribution volumes were detected, the clearance rate of the administered leucine was increased by 61%, and the turnover rate increased by 124%. These data support the view of an increased leucine oxidation in rats bearing the Walker-256 carcinosarcoma.

L-[1-^{14}C]Leucine oxidation *in vitro*

The first irreversible step in leucine catabolism is the reaction catalysed by branched-chain oxo acid dehydrogenase (EC 1.2.4.4), which results in the formation of CO_2 from C-1 of leucine. Measurement of the rate of $^{14}CO_2$ production from L-[1-^{14}C]leucine therefore indicates the flux of the amino acid into the catabolic pathway. In all cases, in the absence of glucose from the medium, the rate of leucine oxidation tended to be lower than in the presence of glucose, although the difference did not reach statistical significance. This observation may be explained by taking into account a lower availability of oxo acids (mainly oxoglutarate) for transamination when exogenous precursors are

Table 2. Utilization of L-[1-^{14}C]leucine by soleus muscles and tumour-tissue slices of control and tumour-bearing rats

For full details, see the Experimental section. The incubation medium contained 0.1 mM-L-leucine and, where stated, 5 mM-glucose. The results are mean values ± S.E.M. for four different animals and represent nmol of L-[1-^{14}C]leucine converted into $^{14}CO_2$ or incorporated into the tissue/90 min per g wet wt. Values that are significantly different by Student's t test from those of the control animals are indicated by $^{**}P < 0.01$.

Rats...	Control		Tumour-bearing			
	Muscle		Muscle		Tumour	
	No glucose	Glucose	No glucose	Glucose	No glucose	Glucose
$^{14}CO_2$	82±7	100±9	128±12**	160±13**	72±5	85±6
^{14}C incorporation into the tissue	96±7	84±6	84±7	81±3	84±9	95±10
Oxidation/incorporation ratio	0.85	1.22	1.52	1.97	0.86	0.89

omitted from the medium. In the muscles of tumour-bearing rats, the oxidation of the amino acid was greater than in the control group, in both the absence and the presence of glucose in the medium (Table 2). Conversely, the incorporation of the tracer into the tissue was unchanged by tumour burden. This resulted in a mean 1.7-fold increase in the oxidation/incorporation ratio in the tumour-bearing group in relation to the control group (Table 2). The ATP content at the end of the different incubations was 3.2 and 2.8 for soleus muscles of control and tumour-bearing animals, respectively, and 0.7 μmol/g fresh wt. of tissue for tumoural tissue. These values were, in all cases, about 80% of those found before the incubation.

The experiments both *in vivo* and *in vitro* support the view that there is an accelerated leucine oxidation in tumour-bearing animals, which may be partially accounted for at the level of skeletal muscle, this tissue being quantitatively the most important site of branched-chain amino acid oxidation. This accelerated leucine oxidation can also be seen in other types of metabolic situations, such as starvation (Buse *et al.*, 1976), diabetes (Buse *et al.*, 1976) or physical exercise (White & Brooks, 1981). The results also support the view that the Walker-256 carcinosarcoma can readily utilize leucine for both oxidation and synthesis. Therefore, both skeletal muscle and tumoural tissue seem to contribute to the enhanced turnover of the amino acid (Table 1). Indeed, tumours act as nitrogen traps (Mider, 1951), retaining amino acids for both oxidation and protein synthesis (Shapot, 1979; Rivera *et al.*, 1988). In addition, leucine, isoleucine and valine have been found to be effectively oxidized in some Morris hepatomas (Wagle *et al.*, 1963). In some cases, the presence of a tumour enhances protein degradation (Lundholm *et al.*, 1976) and decreases protein synthesis (Lundholm *et al.*, 1979) in the host tissues. Lundholm *et al.* (1982) found that the incorporation rate *in vivo* of [^{14}C]leucine into muscle protein was significantly decreased and the fractional degradation rate of protein was increased in cancer patients. On these lines, the carbon skeletons arising from transamination of branched-chain amino acids provide a source of fuel for the skeletal muscle, whereas alanine and glutamine arising from muscle protein degradation are subsequently used by the liver for gluconeogenesis, further increasing the host's metabolic burden (Argilés & Azcón-Bieto, 1988).

Concluding remarks

It has been previously reported that amino acids may play an important role in cell proliferation (Linder-Horowitz *et al.*, 1969). Similarly, energy production resulting from the oxidation of these compounds is envisaged as occurring simultaneously with, and in addition to, that derived from glycolysis. In addition to their role as energy substrates, they can serve as amide- or amino-nitrogen donors in reactions leading to the synthesis of important metabolites, including the pyrimidine, purine and nicotinamide nucleotides (Tate & Meister, 1973), which are absolutely vital for tumour cell proliferation.

In the host, the metabolic signal that stimulates branched-chain amino acid catabolism in muscle during tumour growth still remains to be determined. Hormonal changes could be involved. Indeed, the concentration of glucocorticoids is increased in tumour-bearing animals (Saez, 1971), this situation favouring both muscular protein degradation and hepatic amino acid uptake (Argilés & Azcón-Bieto, 1988; Argilés & López-Soriano, 1990). The insulin resistance that often accompanies cancer-bearing states could also be involved, since it could lead to a turn-down of protein synthesis, as is often seen in metabolic situations such as diabetes or undernutrition (Millward *et al.*, 1976). The role of ketone bodies as important metabolic signals has often been emphasized (Robinson & Williamson, 1980); however, in tumour-bearing animals the concentrations of these metabolites are still high after a long starvation period (Rofe *et al.*, 1986), and this fact, rather than contributing to explaining amino acid release by skeletal muscle, could actually confer an advantage on the host by depressing oxidation of branched-chain amino acid in muscle.

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