THE IN VIVO EFFECTS OF LEUCINE DEHYDROGENASE FROM *BACILLUS SPHAERICUS*

J. ROBERTS, F. A. SCHMID and K. TAKAI
Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

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1. Introduction

It has been reported that leucine dehydrogenase (L-leucine:NAD oxidoreductase [deaminating] EC class 1.4.1) from *Bacillus sphaericus* IFO 3525 was highly inhibitory to Ehrlich ascites carcinoma in vivo [1]. The present report describes experiments which indicate that a homogeneous preparation of this enzyme did not inhibit growth of Ehrlich ascites carcinoma or the Taper liver tumor. Furthermore, no significant change in plasma leucine levels was observed in mice receiving injections of this enzyme. The lack of in vivo effectiveness of *B. sphaericus* leucine dehydrogenase may be explained on the basis of its pH-activity profile and equilibrium considerations.

2. Experimental

Leucine dehydrogenase (L-leucine:NAD oxidoreductase [deaminating] EC class 1.4.1) which was purified to homogeneity as previously described [2] was a gift from Dr. Kenji Soda, Kyoto University. The enzyme was dialyzed overnight at 4°C against 500 vol. of 0.01 M sodium phosphate buffer (pH 7.6) containing 0.9% NaCl. The concentration of the enzyme solution following dialysis was adjusted to 0.8 mg enzyme protein/ml. After dialysis the enzyme was stable for at least 4 days at 4°C. Fresh enzyme solution for injection was prepared every third day and stored at 4°C.

Leucine dehydrogenase was assayed by measuring the rate of increase in absorbance at 340 nm resulting from reduction of NAD. The reaction mixture consisted of 125 μmoles glycine–KCl–KOH buffer (pH 11.3), 10 μmoles L-leucine and 1.25 μmoles β-NAD in a total vol. of 0.8 ml. The reaction was initiated by the addition of 5 μl enzyme solution (containing 16 μg protein) and the increase in absorbance at 340 nm was followed on a Gilford 240 spectrophotometer. The temperature was 25°C. One unit (U) of activity is the amount of enzyme which causes the increase in 1.0 in absorbance per minute at 340 nm.

The ascites forms of Ehrlich carcinoma and Taper liver tumor were carried in $BD2F_1$ mice. Female mice weighing 19–22 g were inoculated intraperitoneally with 10^7 cells on day 0 and treated for 14 days with leucine dehydrogenase, beginning on day 1.

Amino acid analyses were performed on a Beckman Model 119 amino acid analyzer using lithium citrate buffers for the acidic and neutral amino acids [3–5]. Blood for determination of plasma amino acids was obtained from mice by the orbital bleeding technique [3]. Special care was taken to minimize amino acid alterations during the processing of blood and plasma samples for amino acid analysis [3].

3. Results and discussion

The leucine dehydrogenase was assayed prior to injection and found to contain 80 units (U) per mg protein. Table 1 shows that daily injection of leucine dehydrogenase for 14 days at 4 mg/kg body weight did not significantly alter the median survival time of mice bearing the Ehrlich ascites carcinoma or the

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Table 1
Effect of leucine dehydrogenase treatment on tumor growth

Tumor	Enzyme dose mg/kg/day	Median survival day of mice (range)	
		Control	Treated
Taper liver*	4	18(14–21)	18(15–33)
Ehrlich carcinoma*	4	20(19–23)	19(18–25)
Ehrlich carcinoma*	4	18(16–19)	16(14–17)
Ehrlich carcinoma*	8	18(16–19)	22(18–24)
Ehrlich carcinoma*	16	18(16–19)	19(13–22)
Ehrlich carcinoma**	4–16	19(16–23)	19(14–25)

* There were 5 mice in each treatment group.
** Since no trend was observed for median survival time as related to dose of leucine dehydrogenase, the 2 saline controls were pooled as one group, compared with the 4 treatment groups (doses 4, 4, 8 and 16 mg/kg/day).

Taper liver tumor. Even at a dose as high as 16 mg/kg/day no antitumor effect was observed (table 1). Inspection of the organs revealed no gross toxicity resulting from treatment of mice with leucine dehydrogenase. Oki et al. [1] reported that treatment of DD mice bearing an Ehrlich ascites carcinoma with *B. sphaericus* leucine dehydrogenase (S.A. = 100) at a dose of 1 or 4 mg/kg/day for 14 days resulted in a greater than 100% increase in survival time in treated animals. It should be noted that in our experiments mice were inoculated with 1×10^7 ascites cells, while Oki et al. used 2×10^6 cells. It is, however, improbable that the greater number of tumor cells is responsible for the therapeutic ineffectiveness of leucine dehydrogenase in our studies, since there was no decrease in plasma leucine levels in enzyme-treated mice (table 2). Oki et al. [1] did not determine the effect of treatment with leucine dehydrogenase on amino acid levels in the host's circulation.

The results described in the present communication appear to be consistent with the kinetical properties of the enzyme and the equilibrium constant of the reaction in vitro. The enzyme requires NAD as an essential cofactor [2] the concentration of which is negligible in serum. We have found that the pH optimum of the reaction is between 10 and 12 and the enzyme activity at physiological pH (7.4) is less than 5% of the activity obtained under the standard assay conditions (pH 11.3). Sanwal and Zink [6] reported the equilibrium constant for the reaction,

Table 2
Effect of leucine dehydrogenase treatment on plasma amino acid levels

Experiment No.	Enzyme dose × days (mg/kg/day)	Plasma leucine concentration (nmoles/ml)			
		Hours post enzyme injection			
		0 (Control)	*1*	*5*	*24*
1*	4 × 1	74	99	81	94
2**	Saline control			90	
	4 × 5			70	
	8 × 5			83	
	16 × 5			76	

* Normal mice.
** Tumor-bearing mice.

$$k' \text{ eq} = \frac{(\text{NADH})\,(\alpha\text{-ketoisocaproate})\,(NH_3)\,(H^+)}{(\text{NAD})\,(\text{L-leucine})}$$

to be 11.1×10^{-14} (moles/l.)^2. Taking into account the physiological pH (7.4) and the concentration of ammonia in serum (approximately 3×10^{-5} M) [7,8], the equilibrium concentration of the other four reactants might be expected to satisfy the equation described below:

$$\frac{(\text{NADH})\text{ eq }(\alpha\text{-ketoisocaproate})\text{ eq}}{(\text{NAD})\text{ eq }(\text{L-leucine})\text{ eq}} = 9.3 \times 10^{-2}.$$

It is apparent that the breakdown of leucine will not occur readily in serum in the presence of *B. sphaericus* leucine dehydrogenase.

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