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Antilymphoma Activity of a Glutaminase-Free L-Asparaginase of Microbial Origin[1] (39844)

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Introduction. L-Asparaginase has been demonstrated to be an effective antilymphoma agent in animals (1, 2) and man (3, 4). The first L-asparaginase used in a clinical trial was isolated from guinea pig serum (5). Since L-asparaginase is not present in large quantities in guinea pig serum, it is not a practical source of the enzyme for routine chemotherapy. Two enzymes isolated from bacterial sources, *Escherichia coli* L-asparaginase EC-2 (6) and the L-asparaginase from *Erwinia carotovora* (7), exhibit useful chemotherapeutic properties and have been used clinically (8). However, the microbial enzymes can cause acute host toxicity including liver and pancreas complications (8) and immunosuppression (9). These side effects were not observed in toxicological studies using monkeys or in one human case receiving L-asparaginase from guinea pig serum (5).

It has been suggested that the side effects of L-asparaginases from microbial sources may be due to their capability to hydrolyze L-glutamine (10, 11). Although L-glutamine is a nonessential amino acid in man, it is ordinarily required by cultured mammalian cells (12). In addition, L-glutamine deprivation in tissue culture leads to breakdown of polyribosomes and subsequent cessation of protein synthesis (13). While L-glutamine is not required in the human diet, certain tissues including lymphoid tissue contain small amounts of L-glutamine synthetase (14) and obtain exogenous L-glutamine from the blood (15).

When L-asparaginase from *E. coli* is added to *in vitro* cultures of human lymphocytes stimulated with phytohemagglutinin, the blastogenic response of the cells is inhibited (16). This effect can be reversed by L-glutamine but not by L-asparagine (16). Similarly, L-glutaminase isolated from *E. coli* exhibits immunosuppressive effects that are also reversed by L-glutamine and not L-asparagine (17). In contrast, however, when agouti serum which contains no L-glutaminase activity was used as a source of L-asparaginase, similar immunosuppression was not observed (18).

Nutritional studies (19) demonstrated that *Vibrio succinogenes*, a cytochrome-containing anaerobic bacterium, could be grown under conditions in which L-asparagine hydrolysis was growth limiting. Subsequently, *V. succinogenes* was shown to produce L-asparaginase constitutively and crude extracts had a low cross-reactivity toward L-glutamine (20). More recently, the L-asparaginase of *V. succinogenes* has been purified to homogeneity and highly purified preparations have been characterized (21). The enzyme was shown to have a K_m of 4.8×10^{-5} *M* for L-asparagine and was found to hydrolyze L-glutamine at a rate only 0.015% of that for L-asparagine (21). This rate is 130- to 600-fold less than that reported for the L-asparaginases of enterobacteria currently in clinical use (11, 22). Immunodiffusion indicated that the L-asparaginase of *V. succinogenes* is immunologically distinct from the chemotherapeutically active L-asparaginase (EC-2) of *E. coli* (21). Thus, the *V. succinogenes* L-asparaginase

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fulfills the requirements for an L-asparaginase that can be obtained in large quantities which would not be expected to exhibit glutaminase-associated toxicity. In this communication, we describe the antilymphoma activity of this enzyme and its clearance time in tumor-bearing mice.

Materials and methods. Animals. Female C3H mice used in these studies were obtained from the National Cancer Institute. Mice were transplanted with the Gardner lymphosarcoma (6C3HED) by subcutaneous implants of minced tumor on the flanks of the animals. All of the mice used weighed between 20 and 25 g.

Asparaginases. Escherichia coli asparaginase (EC-2) was obtained from Squibb, Inc. Vibrio succinogenes asparaginase was purified as previously described (21).

Antilymphoma activity. The effect of homogeneous L-asparaginase preparations on the 6C3HED lymphosarcoma in C3H mice was determined as follows. Mice in the L-asparaginase-treated groups were given a total of 2.67 IU of enzymes in 0.01 *M* potassium phosphate buffer, pH 7.0. The specific activity of the homogeneous *V. succinogenes* L-asparaginase preparation was 202 IU/mg of protein (1 IU = amount of enzyme that catalyzes the formation of 1 μmole of ammonia/min). Two ip injections per day for 4 days were administered beginning 12 days after tumor implantation. Control mice were given buffer alone on the same regimen of injections. The data shown (Fig. 1) are for seven mice in the *V. succinogenes* L-asparaginase- and buffer-injected groups, eight mice in the EC-2-treated group, and four mice treated with heat-inactivated (60° for 1 hr) L-asparaginase from *V. succinogenes*.

Clearance time from the blood stream. Blood levels of L-asparaginase activity in mice after ip injections of L-asparaginase from *V. succinogenes* (sp act = 202 IU/mg of protein) were determined as follows. Blood removed from the tail vein at the various times was assayed for L-asparaginase by the procedure of Broome (23), except that 0.1 *M* sodium borate buffer (pH 8.5) with 20 m*M* EDTA was used and the reaction was stopped with 5% trichloroacetic acid. Each of the curves (Fig. 2) for the

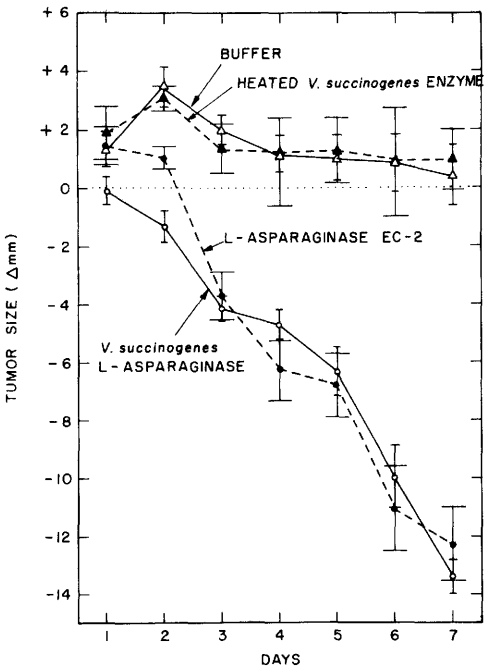

FIG. 1. Effect of L-asparaginase on the 6C3HED lymphosarcoma in C3H mice. Each point designates mean tumor change measured along three axes with calipers, and the bars denote the standard error of the mean. Prior to treatment the average tumor size for each group of animals was 12.6 mm (buffer), 12.6 mm (heated *V. succinogenes* enzyme), 12.4 mm (L-asparaginase EC-2), and 13.4 mm (*V. succinogenes* L-asparaginase).

6C3HED mice represents the average value for two animals; that for the C3H mice is the average for four animals. Linear regression analysis was employed to fit each curve. The resulting correlation coefficients were >0.995 for the 6C3HED animals and at least 0.90 for the C3H.

Results and Discussion. In preliminary tests, five C3H mice with transplanted 6C3HED lymphosarcoma received ip injections of a partially purified preparation of *V. succinogens* L-asparaginase (sp act = 81 IU/mg of protein). Five days after the first injection, significant regression was observed, whereas the mean tumor size of five mice injected with buffer alone had increased markedly.

In the subsequent studies, the antilymphoma activity of homogeneous L-asparaginase from *V. succinogenes* was compared to the effect of the EC-2 enzyme from *E. coli*

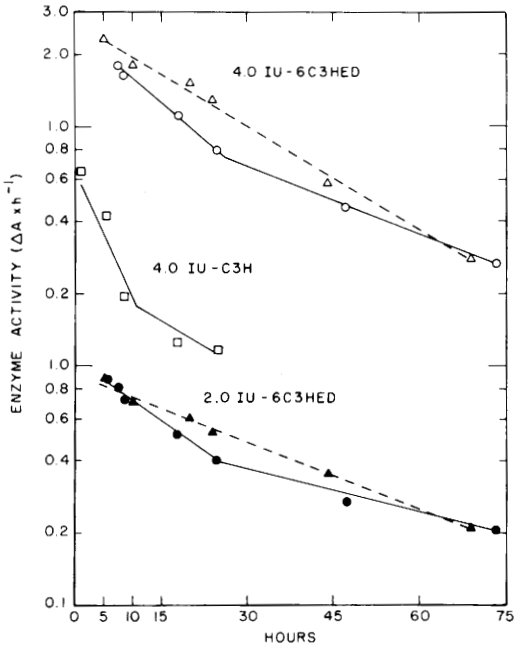

FIG. 2. Blood levels of L-asparaginase activity in mice after ip injections of L-asparaginase from *V. succinogenes* (sp act = 202 IU/mg of protein). Each point represents the average of two animals, except in the C3H group where each point represents the average of four animals. Each curve was fitted by a linear regression analysis.

on the 6C3HED lymphosarcoma of C3H mice (Fig. 1). The two L-asparaginase groups exhibited similar rates of regression, but, on the second day, the group treated with the *V. succinogenes* enzyme seemed to respond more rapidly to treatment than that treated with the *E. coli* enzyme. By the seventh day, all tumors had regressed in the enzyme-treated animals, and tumors had not returned at 30 days, at which time the experiment was terminated. In contrast, control animals given buffer alone or animals given heat-inactivated L-asparaginase from *V. succinogenes* showed a small increase in tumor size the first 2 days; all of these animals died within 30 days of the beginning of the experiment. The overall results of this experiment indicate that the L-asparaginase from *V. succinogenes* was at least as effective as the EC-2 enzyme from *E. coli* in causing tumor regression.

The blood levels of L-asparaginase in mice bearing 6C3HED lymphosarcomas were determined after ip injection of the *V. succinogenes* L-asparaginase (Fig. 2). Enzyme activity in the blood of the tumor-bearing mice reached a maximum at 5 to 7.5 hr and gradually fell during the remainder of the experiment. In contrast, the activity in the C3H control group reached a maximum within 1 hr; however, the maximal activity was much lower than that of the 6C3HED groups which received 4.0 IU of enzyme. In some of the animals, a biphasic activity curve is seen (Fig. 2). Such a biphasic response has been observed for the plasma levels of L-asparaginase in human cancer patients after the administration of some preparations of *E. coli* L-asparaginase (24). The half-life of the *V. succinogenes* L-asparaginase in the blood stream of mice was determined from the data shown in Fig. 2. Depending upon the phase of the curve used in the calculation, it varied between 17 and 42 hr in the 6C3HED group. By comparison, in normal C3H mice, the enzyme was cleared from the blood stream more rapidly; a half-life of approximately 6 hr was calculated for the first phase and 22 hr for the second phase of the activity curve. Other investigators who have used this same animal model system have observed large variations between normal and tumor-bearing mice for the time required to reach maximal levels of enzyme and the half-life of enzyme activity. These variations can be attributed to the fact that the Gardner lymphosarcoma is infected with the LDH virus (25). It has been reported that L-asparaginase EC-2 has a half-life of approximately 3 hr in C3H mice (26) and a half-life of 19–24 hr in tumor-bearing mice with the 6C3HED lymphosarcoma (25). Half-lives up to 23 hr have been reported for the EC-2 enzyme in man (24).

These experiments clearly demonstrate that the L-asparaginase from *V. succinogenes* is a potent antilymphoma agent. The immunosuppressive effects of this enzyme are currently under investigation, and experiments thus far suggest that it is significantly less immunosuppressive than other L-asparaginases currently in clinical use (J. A. Distasio, unpublished results). The nearly undetectable L-glutaminase activity, together with its antigenic specificity and low K_m (21), suggest that the *V. succinogenes* L-asparaginase is an ideal candidate for thor-

ough clinical evaluation.

Summary. The antilymphoma activity and blood clearance behavior of the glutaminase-free L-asparaginase from *Vibrio succinogenes* was tested in C3H mice with transplanted 6C3HED lymphosarcoma. Seven animals in which tumors had been implanted 12 days earlier each received two ip injections of 0.33 IU of homogeneous enzyme per day over a 4-day period. All animals appeared to be in complete regression 3 days after the injections were terminated and showed regression profiles that were at least comparable to those obtained with a second group of 6C3HED mice injected with equivalent levels of EC-2 L-asparaginase. The tumors did not return within 30 days in the enzyme-treated groups, whereas all animals injected with buffer or heat-inactivated *V. succinogenes* enzyme died during this period. Half-lives of 17–42 hr were observed for the *V. succinogenes* L-asparaginase in the blood stream of eight tumor-bearing mice. These results demonstrate that the L-asparaginase from *V. succinogenes* is an effective antilymphoma agent.

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