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A Comparison of Concurrent and Delayed Tests for Antitumor Activity of L-Asparaginase.* (31793)

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Two methods of testing have been used to measure the sensitivity of tumors to various L-asparaginase preparations(1-9). One of these which we may refer to as the concurrent test(1-6), measures the ability of the agent to repress the development of a tumor when a given number of tumor cells are inoculated. In this test the agent is administered within several hours after inoculation of the tumor. The second test (7-9), on the other hand, measures the ability of the agent to induce regression of an established tumor. This we may call the delayed test since the agent is not injected until the inoculated cells have developed into a measurable tumor. It might be assumed that if an agent can prevent the development of a tumor, it would also be efficient in bringing about regression of an established growth and, likewise, an agent which had no effect on tumor development would be inefficient in producing regression of an established transplant. These assumptions have been found to be incorrect in our work involving the effect of L-asparaginase from different sources on various tumors. The data reported here show that the result of one type of test cannot predict the outcome of the other. In fact, the two types of tests gave dissimilar results in each of 3 tumor systems tested.

Materials and methods. Guinea pig serum (GPS) was either obtained commercially or collected from Hartley guinea pigs by cardiac puncture under ether anesthesia. *Escherichia coli* L-asparaginase (EC-2) was purified according to the procedures described separately(10). Guinea pig serum L-asparaginase was administered as whole GPS but EC-2 was purified to some degree since the crude extract of *E. coli* is toxic. It was found, however, that the degree of purification of the enzyme did not alter the ability to detect its antilymphoma activity so long as the toxic materials were removed. Tumor cells were injected (EARAD1 into (C57BL/6 × A)F_1 mice (11), 6C3HED into C3H/HeJ mice(12), or P1798 into BALB/c mice(13)) subcutaneously in the flank of shaved female mice an hour before (concurrent test) or 7 days before (delayed test) the administration of L-asparaginase. The animals were treated

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TABLE I. Comparison of Concurrent and Delayed Tests of L-Asparaginase on Leukemia, EARAD1.

Treatment	Enzyme units[a]	Concurrent test[b,c]		Delayed test[b,d]	
		Time to tumor development (days)	Survival time (days)	Tumor reduction	Survival time (days)
None	0	5.8	16.8	0	16.6
EC-2	50	5.8	17.0	7.0	18.0
GPS	50	9.3	22.8	0	16.5
EC-2	200	6.3	17.7	10.0[e]	21.0
GPS	200	14.0[f]	37.3[f]	7.0	20.8
EC-2	2000	7.5	18.8	11.0[e]	37.0[g]

[a] One unit of activity is that amount of enzyme which will hydrolyze one micromole of L-asparagine in one hour at the maximum rate.
[b] The values are averages of 5 mice in control groups and of 4 mice in experimental groups.
[c] 1.0×10^6 cells were in tumor inoculum.
[d] 3.5×10^6 cells were in tumor inoculum.
[e] Complete remission.
[f] One mouse of the 4 tested did not develop a tumor and is not included in the average.
[g] One mouse of the 4 tested was cured and is not included in the average.

with a single intraperitoneal injection of the L-asparaginase sample. The average tumor diameter at the beginning of the delayed tests was 10-12 mm (EARAD1), 5-6 mm (P1798) or 9-10 mm (6C3HED). Tumor measurements were recorded daily. An animal was considered cured if it lived free of tumor symptoms for at least 60 days after complete remission. Mice in the concurrent tests were examined regularly for tumor development.

Results and discussion. It can be seen in Table I that 50 units of L-asaparginase from GPS causes a significant delay in tumor development as well as a prolonged survival time when administered concurrently (within an hour) with the inoculum. However, there is no effect on the established tumor or on the survival time when this therapy is given 7 days after the tumor cell inoculum. Conversely, the same amount of L-asparaginase (EC-2) from *E. coli* has no observable antitumor activity when tested concurrently but it causes a substantial reduction in the established tumor when administered 7 days after inoculation. Even 2000 units of EC-2 show little antilymphoma activity in the concurrent test. However, this dosage given to each of 4 mice bearing a 7-day transplant of EARAD1 resulted in one cure and the survival time of the other 3 mice was double that of the untreated controls.

Table II shows that the striking differences in the 2 types of tests observed in the experiments on EARAD1 are also observed when the tests are performed on the 6C3HED and the P1798 lymphosarcomas. As little as 108 units of GPS L-asparaginase administered within an hour after the inoculum prevented the development of 6C3HED in 2 of the mice and greatly delayed the appearance of the tumor in the third. Nine hundred units of EC-2, however, delayed tumor development only slightly when tested concurrently but cured all 3 mice bearing 9 mm tumors at the time of treatment.

P1798 development in BALB/c mice was also greatly delayed by small amounts of GPS L-asparaginase but not by the same dosage of EC-2 (Table II). When the concurrent test was used, 300 units of EC-2 gave no significant retardation and as much as 2000 units only delayed the development of a measurable tumor by 4 days. However, when 300 units of GPS L-asparaginase was given, no measurable tumor developed until 14 days after those of the control animals. When tumor bearing mice were treated complete remission was induced by 300 units of L-asparaginase from either source, but those treated with EC-2 had significantly longer survival times.

L-asparaginase from yeast has been reported to be devoid of antitumor activity when tested concurrently(5). In view of the rapid clearance of this enzyme from the

TABLE II. The Effect of L-Asparaginase on 6C3HED and P1798 Lymphosarcomas.

Tumor	Enzyme	Enzyme units[a]	Concurrent test[b,c]		Delayed test[b,c]	
			Time to tumor development (days)	Survival time (days)	Tumor reduction (mm)	Survival time (days)
6C3HED		0	5.0	17.0	0	17.0
	GPS	108	16.0[d]	33.0[d]	4.0	22.7
	EC-2	900	7.7	20.3	9.0[e]	all cured
P1798		0	7.3	24.0	0	24.0
	GPS	100	19.0[f]	36.5[f]	3.7	28.7
	GPS	300	21.3	36.0	5.4[e]	30.7
	EC-2	300	8.7	24.0	5.3[e]	36.0
	EC-2	2000	11.3	27.0	5.8[e]	43.3

[a] One unit of activity is that amount of enzyme which will hydrolyze one micromole of L-asparagine in one hour at the maximum rate.
[b] Values are average of 3 mice.
[c] 3.6×10^6 cells were in tumor inoculum.
[d] Two of the 3 mice did not develop tumors and are not included in the average.
[e] Complete remission.
[f] One of the 3 mice did not develop a tumor and is not included in the average.

circulation(5) it may have no effect on an established tumor either, but on the other hand, it may be similar to EC-2 in being quite promising in the delayed test. The details of the different manifestations of GPS and EC-2 to the antilymphoma action of L-asparaginase are discussed elsewhere(14).

An agent may appear to have antitumor activity against an established tumor but not towards a fresh inoculum if the material is unstable in the body or if the material is rapidly cleared from the circulation. Further, the freshly inoculated cells may be in a lag phase of growth and consequently have metabolic requirements different from those of the established tumor. This would be of particular importance in the case of agents that are unstable in the body or cleared before the inoculated cells enter a growth cycle.

The remarkable difference in the results obtained with these two samples of L-asparaginase may not be a special case, but rather a general problem in the evaluation of chemotherapeutic agents. The method described in the standard protocols for screening for the Cancer Chemotherapy National Service Center(15) is a combination of the two tests. The treatment is started 24 hours after the tumor cells are inoculated and is continued with daily injections for a week or more. This type of testing is probably adequate to detect any antitumor activity of either type but requires large amounts of material. The delayed test, however, requires smaller amounts of material and regression can be detected in a short time(7,8). From the data reported here it appears that the method used to detect antitumor activity is critical. The chemotherapeutic activity of EC-2 would not have been detected by the exclusive use of the concurrent test. As the aim of research in the chemotherapy of malignant disease is to find agents which will produce regression and cure of the disease in man, the most pertinent tests are those performed on established tumors. Thus, the use of the delayed test undoubtedly has a primary role in the evaluation of new agents.

Summary. Delayed testing is compared to concurrent testing for the evaluation of chemotherapeutic agents using samples of L-asparaginase as test materials. A large difference in the detection of the antitumor activity of the agents was found, depending upon the method of testing. Guinea pig serum L-asparaginase was quite effective in repressing tumor development when administered within an hour of tumor inoclation while an L-asparaginase (EC-2) from *Escherichia coli* was not. The delayed test (treatment given 7 days after tumor inoculation) however, showed that EC-2 was able to induce complete remission of an established transplant of each of the 3 lymphoid tumors tested (EARAD1, 6C3HED, and P1798).

These results draw attention to the value of the delayed test for the screening of potential chemotherapeutic agents.

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Effect of Splenectomy on Primary and Secondary Response to Sheep Erythrocytes in Rats. (31794)

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Rowley(1) has demonstrated that hemolysin production in the splenectomized rat is a function of the dose and route of administration of red cell antigens. A small dose of sheep red blood cells (SRBC) injected intravenously led to the production of minimal amounts of antibody. He also found that the time of antigen administration after splenectomy did not change these results. However, splenectomy of *S. typhi* immunized rats showed somewhat different results. Rats splenectomized before *S. typhi* injection showed the same antibody depression as seen in rats splenectomized before SRBC injection. But when the spleen was removed 14 days *after S. typhi* injection, there was little effect on the agglutinin response(2,3).

It has recently been shown that antibody-containing and antibody-producing cells migrate from the spleen into the blood during late stages of the primary immune response (4,5). These cells will naturally reenter lymphoid organs in the course of their migration. It seems reasonable to think that if the cells which are found in the circulation are immunologically competent, removal of the spleen 7 or 14 days after antigen should not inhibit the response to that antigen. It also seems possible that if splenectomy were several days after antigen, some antigen might be 'processed' and might prepare the animal for an anamnestic response even if the spleen has been removed before the second injection of antigen.

Therefore the purpose of the experiments reported here was 2-fold: (1) To see if splenectomy 7 or 14 days after SRBC antigen would cause decreased production of hemolysins, and (2) To see if splenectomy 7 or 14 days after SRBC antigen would render rats unable to respond to a subsequent injection of the same antigen.

Materials and methods. Male albino Sprague-Dawley rats weighing 200-300 g were used. They were maintained on Purina rat pellets and water *ad libitum*.

A 1% suspension of sheep red blood cells (SRBC) was prepared by washing whole sheep blood 3 times and diluting in physiologic saline so that 1 ml of SRBC lysed with 4 ml of water gave a reading of 150 in a Klett-Summerson colorimeter with a 540 mμ filter. One ml of antigen was injected intravenously (i.v.) into the lateral tail vein of each rat while the animal was under light