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SUPPRESSION OF SKIN HYPERSENSITIVITY AND ANTIBODY FORMATION BY L-ASPARAGINASE

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Rabbits immunized with BSA in Freund's adjuvant were treated with different doses of L-asparaginase. The administration of this enzyme was started on the day of immunization and continued for 21 days. To measure the immunologic responses, weekly skin tests and quantitative antibody determinations were done. It was found that L-asparaginase in large doses (1000 IU/kg/day) suppressed both antibody formation and delayed hypersensitivity. With smaller doses, the suppression was partial and antibodies against L-asparaginase itself appeared in the serum, leading to a rapid elimination of the enzyme from the system by the end of the 3rd week. This rapid clearance of asparaginase in the latter part of its administration in small doses is probably due to the neutralizing effect of the antibodies, or rapid elimination of the antigen-antibody complexes, or a combination of the two. The possible mechanism and clinical significance of these findings was discussed.

Various chemotherapeutic agents, used in cancer, have proved to be immunosuppressive (1-7). With the increasing evidence of the significant role played by immunity in the control of malignant disease (8), it becomes very desirable to find an agent which will be devoid of immunosuppression and yet effective against cancer.

Recently, L-asparaginase (L-asparagine amidohydrolase) which belongs to a new class of therapeutic agents has given promising results in both animal tumors and human leukemia (9-17). In the present study, the effect of L-asparaginase on the immune responses was investigated in rabbits. This enzyme was found to be immunosuppressive. It is interesting to note that, while large doses proved to be immunosuppressive, antibody against L-asparaginase was detected in animals receiving small amounts of this agent. To our knowledge, this is the first example of an immunosuppressive agent reported in literature which belongs to the category of enzymes. It is antigenic in small doses. These observations, in addition to yielding information regarding the immune responses, may have important implications from the therapeutic point of view.

MATERIALS AND METHODS

L-Asparaginase. *Escherichia coli* L-asparaginase prepared at the Wadley Institutes of Molecular

Medicine, which is being given clinically, was used in these experiments. This preparation had a specific activity of 358 units/mg of protein. The enzyme activity was determined according to the method described by Roberts *et al.* (18) and expressed in international units (IU). The protein determinations were done by the biuret method (19). L-Asparaginase was administered intravenously (i.v.) in varying doses for 21 days, starting on the day of immunization (Tables I and II).

Animals. New Zealand White rabbits, both male and female, weighing between 1740 and 2200 g, were used. The animals were fed Purina rabbit food and their intake and body weight were measured daily. Initial and weekly absolute lymphocyte counts were done by the standard methods.

Immunization. The animals were immunized against BSA by injecting 0.2 ml of complete Freund's adjuvant containing 5 mg BSA into each footpad and the neck giving a total of 1 ml/rabbit.

Skin test. All the rabbits were skin tested by the intradermal injection of 30 μ g of BSA in 0.1/ml of N saline. Arthus reactivity was determined by measuring the area of erythema and induration at 4 hr and the delayed hypersensitivity at 24 hr (Table II).

Antibody determination. Blood samples were withdrawn from the ear vein at weekly intervals. The sera were stored at -20°C until they were used. The antibody was demonstrated by the precipitation test and optimal proportions of the antigen and antibody were determined (20).

Quantitative determinations of the antibody against BSA and L-asparaginase were done by ultraviolet spectroscopy at 280 nm according to the method described by Eisen (21) and Gitlin (22). To describe the procedure briefly, specific precipitates were obtained in the region of slight antibody excess by adding a known amount of BSA or L-asparaginase in 1 ml of N saline to 1 ml of rabbit serum. The mixture was allowed to react at 37°C for 1 hr and then placed at 1°C for 24 hr. The precipitate was separated by centrifuging at 3000 rpm for 30 min at 1°C and the supernatant tested for the presence of antigen or antibody. The precipitate was washed three times at 1°C and finally dissolved in 0.1 N acetic acid for analysis.

RESULTS

General effects of L-asparaginase. A loss in the body weight was observed in all the rabbits receiving L-asparaginase in doses of 10 IU/kg or higher (Table I). The reduction in weight increased with the increase in dose. There was a proportional loss of appetite. Following cessation of the therapy, the appetite returned to normal and the animals started gaining weight. One animal receiving 1000 IU of L-asparaginase/kg died on the 4th day. There was a slight decrease in the lymphocyte counts of some of the rabbits receiving asparaginase but the difference was not statistically significant from the pretreatment level.

Effect of L-asparaginase on antibody formation. Quantitative determinations of serum antibody levels along with the dose of L-asparaginase given and the serum asparaginase levels are presented in Table I. A good antibody response to BSA was observed in the control group. A decline in antibody formation was observed in the different groups of rabbits as the dose of L-asparaginase increased from 1 IU/kg to 50 IU/kg. Only two of the four animals receiving 100 IU/kg of L-asparaginase and one of the group receiving 500 IU/kg showed a weak antibody response, and the rest failed to develop detectable antibodies against BSA. A further increase in the dose to

1000 IU/kg completely suppressed antibody formation. One of the rabbits in this group died on the 4th day. The animal became weak and developed paralysis of all limbs before death.

It was interesting to observe that all the animals receiving 10 IU/kg, and some of the animals receiving 50 IU/kg and 100 IU/kg, while exhibiting a varying degree of immunosuppression against BSA, showed detectable antibody to L-asparaginase itself on days 35 and 42, i.e., 2 and 3 weeks after the administration of L-asparaginase was stopped (Table I). Maximum antibody to asparaginase was found in the group that received 10 IU/kg. With the increase in dose of L-asparaginase the antibody against this enzyme also decreased and none was detected in the animals that were given 500 IU and 1000 IU/kg.

Serum asparaginase determinations were done on days 7, 14 and 21 before the administration of daily doses (approximately 24 hr after the previous dose), and on day 28 to detect any residual enzyme (Table I). No asparaginase was found in the sera of rabbits that received 1 IU/kg. The animals that were given 10 IU, 50 IU and 100 IU/kg showed enzyme in their sera on days 7 and 14, but none on day 21. The enzyme levels on day 14 were relatively lower as compared to those on day 7 in these rabbits. Fairly constant levels of asparaginase were found in animals given doses of 500 IU and 1000 IU/kg, on days 7, 14 and 21. The enzyme disappeared completely from the serum by the 28th day, i.e., 7 days after the last injection.

Skin reactivity. Table II summarizes both Arthus and delayed skin reactions, which are expressed as the diameters in mm. The skin tests were done on days 7, 14, 21, 28, 35 and 42 following immunization with BSA. We did not perform any histologic studies on the test sites, but it has been demonstrated histologically that skin reactions to BSA show an Arthus type of reactivity at 4 hr and a delayed reaction at 24 hr (23). Although reactions up to 2 mm have been recorded, it is felt that the ones that are 3 mm or smaller may not be significant.

Complete suppression of both Arthus and delayed reactivity was seen with the largest dose of L-asparaginase (1000 IU/kg). Partial suppression of the skin reactions was observed with the smaller doses (1 IU/kg, 10 IU/kg, 50 IU/kg, 100 IU/kg and 500 IU/kg). The decline in skin

TABLE I
Effect of L-asparaginase on antibody formation

L-Asparaginase Administered	Rabbit No.	Antibody to BSA						Atb. to Asp.		Serum Asp. Level				Body Weight		
		Day						Day		Day				Day		
		7	14	21	28	35	42	35	42	7	14	21	28	0	21	42
		$\mu\text{g N/ml}$								IU/ml				g		
1 IU/kg i.v. $\times$ 21 days	1	58	211	362	876	1238	1280							1930	1980	2160
	2	77	260	405	1008	1525	1675							1870	1970	2110
	3	80	230	405	768	1438	1655							2050	2300	2470
	4	62	192	369	856	1355	1390							1950	2030	2200
	Average	69	223	385	877	1389	1500							1950	2070	2235
	5	40	138	280	615	810	933							1760	1940	2190
	6	95	310	428	930	1452	1614							1820	1970	2140
	7	75	255	346	978	1526	1588							1740	1870	2060
	8	52	275	395	823	1230	1466							1950	2110	2290
	Average	66	245	362	837	1255	1400							1818	1973	2170
10 IU/kg i.v. $\times$ 21 days	9	30	263	269	636	1054	1152	137	148	0.24	0.16			1860	1610	2110
	10	44	125	315	710	938	1220	90	110	0.18	0.2			1900	1830	2230
	11		20	47	51	40	52	123	122	0.16	0.12			1850	1700	2160
	12		94	222	446	665	873	75	90	0.28	0.14			1880	1630	2090
	Average	18	126	213	461	674	824	106	118	0.21	0.15			1873	1693	2148
50 IU/kg i.v. $\times$ 21 days	13	35	82	172	211	330	622	42		0.4	0.38			1980	1950	2140
	14		65	152	217	450	880	40		0.56	0.26			1980	1730	1870
	15		50	120	198	370	456			0.5	0.4			1890	1680	1840
	16	42	78	185	243	510	864	75	30	0.48	0.32			1760	1670	1810
	Average	19	69	157	217	415	706	39	8	0.48	0.34			1903	1758	1915
100 IU/kg i.v. $\times$ 21 days	17		20	30	38	30	35	51	35	1.6	1.2			1880	1480	2060
	18							45		1.8	1.0			1880	1220	1970
	19		42	50	35	30	45			1.2	0.8			1790	1260	1720
	20									2.0	1.0			1940	1560	2050
	Average		16	20	18	15	20	24	9	1.65	1			1873	1380	1950
500 IU/kg i.v. $\times$ 21 days	21		65	45	35	48	150			3.8	4.0	3.0		1800	1400	1690
	22									3.7	3.6	3.8		1760	1580	1660
	23									2.4	3.8	3.2		1860	1490	1720
	24									3.8	3.4	4.0		1940	1520	1730
	Average		16	11	9	12	38			3.4	3.7	3.5		1840	1498	1700
1000 IU/kg i.v. $\times$ 21 days	25									6.2	5.8	5.4		2050	1630	1970
	26									4.8	5.6	5.2		2130	1540	1920
	27		Dead							4.1	5.2	4.6		1740	1320	1590
	28															
	Average									5.03	5.53	5.06		1973	1497	1827

* Atb., Antibody.
Asp, L-Asparaginase.

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reactions increased with the increase in dose even within this range. Some of the animals that received 100 IU/kg and 500 IU/kg, with complete suppression of Arthus reactivity and corresponding absence of antibody, still showed delayed reactions.

DISCUSSION

The inhibition of phytohemagglutinin (PHA)-stimulated lymphocyte blastogenesis by L-asparaginase (24) and the better response of patients with leukemia of lymphatic origin (17, 25) suggest that this agent may be immunosuppressive. The present study revealed that this enzyme inhibits the development of antibodies and delayed hypersensitivity to BSA if it is administered for 21 days starting on the day of immunization. The control group of animals showed good immediate and delayed hypersensitivity to BSA with the method of sensitization employed. The observation that immunosuppression is caused by asparaginase, which belongs to an entirely different class from the conventional agents used in cancer treatment, has therapeutic significance, since it thus can be placed in the same category from the immunologic point of view as other anti-tumor agents (1-7).

L-Asparaginase is administered parenterally. It is protein in nature and therefore is potentially antigenic. Antibody against this enzyme has been demonstrated in patients on asparaginase therapy (17, 26). Among 13 patients reported earlier, only one developed antibody to asparaginase (26). This patient received an average of 14 IU/kg/day, which was the smallest dose in that group of patients. The rest of the cases received doses varying from 236 IU/kg/day to 5000 IU/kg/day. In the light of the present report, the absence of antibody in the 12 cases that received a higher dose can be explained on the basis of immunosuppression.

The highest antibody level against asparaginase was seen in the rabbits that received 10 IU/kg/day and complete suppression was observed with doses of 500 IU and 1000 IU/kg. The antibody to asparaginase has been shown to be neutralizing in nature (26). The absence of asparaginase on day 21 in the serum of rabbits that received lower doses (10 IU, 50 IU and 100 IU/kg/day) can be explained on the basis of neutralization by the antibody, a rapid elimination of the antigen-antibody complexes, or a combination of the

two factors. There was no detectable antibody in three rabbits belonging to the groups that received 50 IU and 100 IU/kg and at the same time no asparaginase could be detected in the serum on day 21. The level of antibody could have been below the limit of detection by the method used, but still significant enough to neutralize asparaginase. At higher dose levels (500 IU and 1000 IU/kg) fairly constant serum enzyme levels were found on days 7, 14 and 21. In view of these findings, if constant serum asparaginase levels are to be maintained and if reactions of hypersensitivity are to be avoided, it will be necessary to administer asparaginase in high doses. On the other hand, these doses will be immunosuppressive. It may be interesting to point out that the dose of asparaginase which has given good results clinically (25), falls within the immunosuppressive range for rabbits (1000 IU to 2000 IU/kg/day), although the species difference should be borne in mind. This observation emphasizes the relationship between immunosuppression and anti-tumor effect.

L-Asparaginase acts by hydrolyzing L-asparagine and thus depleting this amino acid. There is 1% to 3% glutaminase activity associated with *E. coli* L-asparaginase, and therefore glutamine is also depleted from the system. It has been observed that both asparagine and glutamine become undetectable in the serum within 45 min after the administration of L-asparaginase and these amino acids remain undetectable for several days following the cessation of therapy (to be published). It is likely that the depletion of these amino acids leads to interference with the formation of antibody protein. Depression of other serum proteins such as fibrinogen, albumin and globulin has been seen during asparaginase therapy (14, 25).

The suppression of delayed hypersensitivity which is mediated by lymphoid cells suggests an effect of the enzyme on this cell type. Similarly, inhibition of blastogenesis of lymphocytes by PHA with asparaginase favors this contention. The only effort made in this direction was the determination of lymphocyte counts, but no statistically significant difference in the number of lymphocytes was observed when initial and 21-day counts were compared. It must be realized that enumeration may be an insensitive technique, and moreover it will not detect any qualitative change in the lymphocytes.

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