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Schedule-dependent Therapeutic Synergism for L-Asparaginase and Methotrexate in Leukemic (L5178Y) Mice[1]

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SUMMARY

In this study therapeutic synergism was observed in combination chemotherapy of leukemia L5178Y with L-asparaginase (A-Ase) plus methotrexate (MTX) over a variety of treatment schedules. Therapeutic synergism occurred when a single dose of A-Ase was followed at varying times (immediately or up to 6 hr later) by daily treatment with MTX. It was also observed when daily treatment with MTX was initiated just prior to or up to 6 hr before a single treatment with A-Ase. Similarly, increases in survival time were observed in leukemic mice when daily treatment with MTX was initiated 4 days prior to a single dose of A-Ase on Day 7. In one instance, the occurrence of therapeutic synergism was dependent upon the sequence of drug administration. Whereas therapeutic synergism was observed when mice bearing L5178Y leukemia were given 5 daily treatments of MTX followed 3 days later by 5 daily treatments of A-Ase; it did not occur when A-Ase preceded MTX on the same schedule. Treatment with MTX was found to be equally effective against leukemia L5178Y and its A-Ase-resistant line.

INTRODUCTION

The therapeutic effectiveness of A-Ase[3] has been observed in lymphomas and leukemias in mice, rats, and dogs and in acute lymphoblastic leukemia in children (2, 7, 12, 13). However, the development of leukemic cell resistance and the immunological response of the host against A-Ase after treatment were found to be limiting factors in the clinical and experimental use of A-Ase (10, 15, 18). The immune response of the host to A-Ase could be prevented by treatment with an immunosuppressive drug (16). Synergism has been reported with alternating combinations of A-Ase plus other chemotherapeutic agents in P1798 leukemia (11). Therapeutic potentiation of A-Ase occurred when vincristine was given before A-Ase or when daunomycin treatment was initiated 2 days prior to A-Ase in mice bearing L5178Y tumor (3). Other therapeutic agents, such as cyclophosphamide and 1,3-bis(2-chloroethyl)-1-nitrosourea, were shown to have additive or synergistic effects in combination with A-Ase against experimental tumors (17, 20). Although previous studies in our laboratory have shown therapeutic synergism between A-Ase and MTX in mice bearing leukemia L5178Y (19), there have been 2 recent reports showing that no therapeutic synergism is observed when these 2 drugs are administered simultaneously (5, 6). The current experiments were conducted to investigate in more detail the parameters of schedule dependency in combination chemotherapy of leukemia L5178Y with A-Ase plus MTX.

MATERIALS AND METHODS

Ten- to 12-week-old C57BL/6 × DBA/2 F_1 (hereafter called $BD2F_1$) or BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice were inoculated s.c. with 10^6 leukemia L5178Y ascites cells which were maintained in DBA/2 mice. The L5178Y/A-Ase variant line is also maintained in DBA/2 mice. Mice bearing 3-day-old L5178Y ascites were treated i.p. with A-Ase (23 i.u.), and the tumor was transplanted into the next generation on Day 14. One set of mice at each generation was challenged with A-Ase (23 i.u.) to verify drug resistance, and the other set of mice was treated and transplants were done into the next generation for maintenance of the resistant line.

MTX was dissolved in 2% sodium bicarbonate solution and was injected at a constant volume of 0.01 ml/g body weight. A-Ase was reconstituted in 0.85% NaCl solution and injected at 0.2 ml/mouse at the doses indicated in the charts and tables.

The schedules of treatment used in these experiments are as follows: (*a*) single treatment with A-Ase on Day 3, 7, or 14 (9 a.m.) followed by daily treatment with MTX (3 p.m.); (*b*) A-Ase on Day 3 (9 a.m.) followed by daily treatment with MTX at 9 a.m., 11 a.m., 1 p.m., or 3 p.m.; (*c*) MTX daily (9 a.m.) from Day 3 followed by A-Ase on Day 3 at 9 a.m., 11 a.m., 1 p.m. or 3 p.m.; (*d*) MTX on Days 3 to 7 (9 a.m.) and A-Ase on Days 10 to 14 (9 a.m.); (*e*) A-Ase on Days 3 to 7 (9 a.m.) followed by MTX on Days 10 to 14 (9 a.m.); and (*f*) treatment of A-Ase variant line with MTX on Day 3 only, every 4 days from Day 3 to death or daily from Day 3 to death. Tumor diameters and body weights were recorded at regular intervals.

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[3] The abbreviations used are: A-Ase, L-asparaginase, NSC 109229; MTX, methotrexate, NSC 740; MST, median survival time; ILS, increase in life-span over the untreated controls; LDH, lactate dehydrogenase.

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RESULTS

Effect of Daily Treatment with MTX, Starting 6 Hr after Single Treatment with A-Ase on the Survival Time of Leukemic Mice

The data summarized in Chart 1 show the effect of single treatment with A-Ase (5 to 108 i.u.) on Day 3, 7, or 14 (at 9 a.m.) followed by daily treatment (at 3 p.m.) with 1.08 mg of MTX per kg from Day 3, 7, or 14 on the survival time of leukemic (L5178Y) mice bearing s.c. tumor. The MST of the untreated controls was 18 days (range, 15 to 23 days). A single treatment with A-Ase (5 to 108 i.u.) alone on Day 3, 7, or 14 gave maximum MST of 27 days. The longest MST for MTX alone was 37.5 days obtained in mice treated from Day 3 with 1.08 mg/kg. The optimal dosage combination of A-Ase plus daily MTX for treatment initiated on Days 3, 7, or 14 gave an MST of 52.5 days, indicating therapeutic synergism for the 2 drugs.

Effect of Time of Injection of Daily MTX Relative to Single Injection with A-Ase on the Survival Time of Leukemic (L5178Y) Mice

Daily Treatment with MTX Starting at Varying Times after A-Ase. Since therapeutic synergism was observed for a single injection of A-Ase on Day 3 at 9 a.m., followed by daily injection of MTX at 3 p.m., another experiment was conducted to study the effect of A-Ase on Day 3 at 9 a.m., followed by MTX daily at 9 a.m., 11 a.m., 1 p.m., or 3 p.m. from Day 3 on the survival time of leukemic mice. The data are summarized in Chart 2. Treatment with a single dose of A-Ase (23 i.u.) given at 9 a.m., 11 a.m., 1 p.m., or 3 p.m. on Day 3 after tumor inoculation produced an ILS of 20 to 27% over the untreated controls. Similarly, daily treatment with MTX, 0.65 mg/kg, at these intervals on Day 3 produced an ILS of 67 to 127%. In the mice treated with the combination of A-Ase (23 i.u.) at 9 a.m. on Day 3 followed by MTX, 0.65 mg/kg daily at 9 a.m., 11 a.m., 1 p.m., or 3 p.m., the ILS ranged from 150 to 193%.

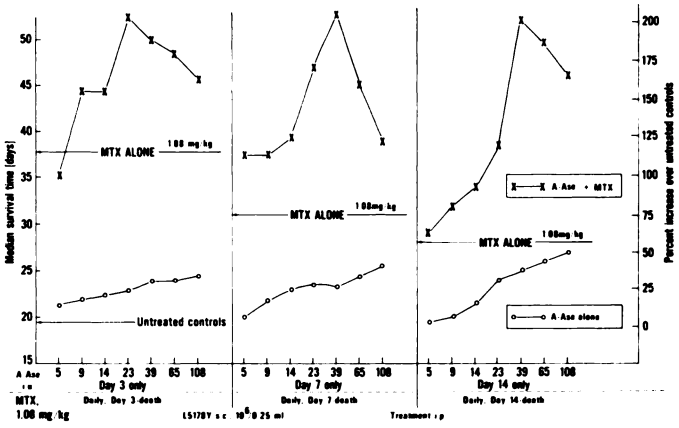

Chart 1. Effect of daily treatment with MTX starting 6 hr after single treatment with A-Ase on the survival time of leukemic (L5178Y) mice; 8 treated mice/group and 20 untreated controls.

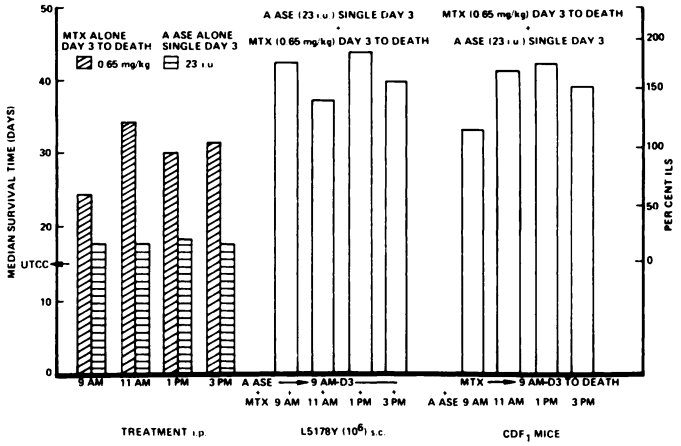

Chart 2. Effect of time of injection of daily MTX relative to single injection of A-Ase on the survival time of leukemic (L5178Y) mice. *UTC*, untreated controls; 8 treated mice/group and 20 untreated controls.

Daily Treatment with MTX Starting at Varying Times Prior to A-Ase. In the same experiment (Chart 2) daily treatment with MTX at 9 a.m. from Day 3, was followed by a single injection of A-Ase at 9 a.m., 11 a.m., 1 p.m., or 3 p.m. on Day 3. In the mice treated daily with MTX at 9 a.m. from Day 3 and given a single dose of 23 i.u. of A-Ase at 9 a.m., 11 a.m., 1 p.m., and 3 p.m. on Day 3, the ILS were 123, 175, 183, and 163%, respectively.

Influence of A-Ase Plus MTX on Tumor Growth at the Site of L5178Y Leukemia Inoculation

Daily Treatment with MTX Starting 6 Hr after Single Treatment with A-Ase on Day 3. The results of an experiment conducted to observe the effect of treatment with A-Ase or MTX alone or in combination on the survival time and tumor growth in leukemic mice (L5178Y) are shown in Table 1 and Chart 3. In the 1st part of the experiment a single i.p. treatment with A-Ase (23 i.u.) at 9 a.m. on Day 3 was followed by daily i.p. treatments with MTX (0.23 to 1.8 mg/kg) at 3 p.m. from Day 3 after s.c. tumor inoculation. The MST of the untreated control mice was 18 days. Treatment with A-Ase (23 i.u.) alone on Day 3 increased the MST to 23 days (ILS, 28%). The maximum survival time (MST, 33.5 days) for MTX alone was observed in mice treated daily with a dose of 0.65 mg/kg. The MST of mice treated with A-Ase (23 i.u.) on Day 3 plus daily MTX (0.23 to 1.8 mg/kg) from Day 3 ranged from 33 to 45 days.

The tumor diameters of mice were measured at regular intervals (Table 1). The tumor grew from 8 to 25 mm in diameter from Days 7 to 17 in the untreated control mice. There was a moderate inhibition and delay in tumor growth in the mice treated with A-Ase or MTX, whereas with the combination of A-Ase plus MTX there was more extensive inhibition of tumor growth. Local tumor growth was not observed up to 25 days in the mice treated with the optimal dose combination of the drugs.

Table 1

Influence of A-Ase plus MTX on tumor growth at the site of L5178Y leukemia inoculation[a]

A-Ase 23 i.u. i.p.	MTX (mg/kg) i.p.	MST[b] (days)	Range (days)	Tumor diameter (mm) on Days 7	11	14	17	19	25
Untreated control		18	17-19	8	16	19	25		
Day 3		23	21-25	0	0	10	19	21	
Day 7		24.5	18-30	0	7	8	18	19	
			Day 3 to death						
	1.8	24	7-28	0	0	6	10	12	14
	1.08	33	28-45	0	0	6	10	12	19
	0.65	33.5	29-38	0	9	9	15	16	19
	0.39	30.5	27-32	0	9	11	18	20	23
	0.23	24.5	22-28	5	12	14	20	22	24
Day 3	1.8	36	24->60[c]	0	0	0	0	0	0
Day 3	1.08	45	24->60[d]	0	0	0	0	0	0
Day 3	0.65	39.5	37-52	0	0	0	0	9	14
Day 3	0.39	39.5	27-41	0	0	8	11	12	17
Day 3	0.23	33	26-35	0	0	8	12	15	20
Day 7	1.8	22	8-26	0	0	0	0	0	5
Day 7	1.08	46.5	41-52	0	0	5	6	10	12
Day 7	0.65	41	35-45	0	0	7	11	11	15
Day 7	0.39	36	28-41	0	0	7	13	15	18
Day 7	0.23	29.5	26-32	0	0	7	14	16	21

[a] $BD2F_1$ male mice were inoculated s.c. with 10^6/0.25 ml L5178Y ascites cells.
[b] Of 8 treated and 20 untreated controls.
[c] Two of 8 survivors.
[d] Three of 8 survivors.

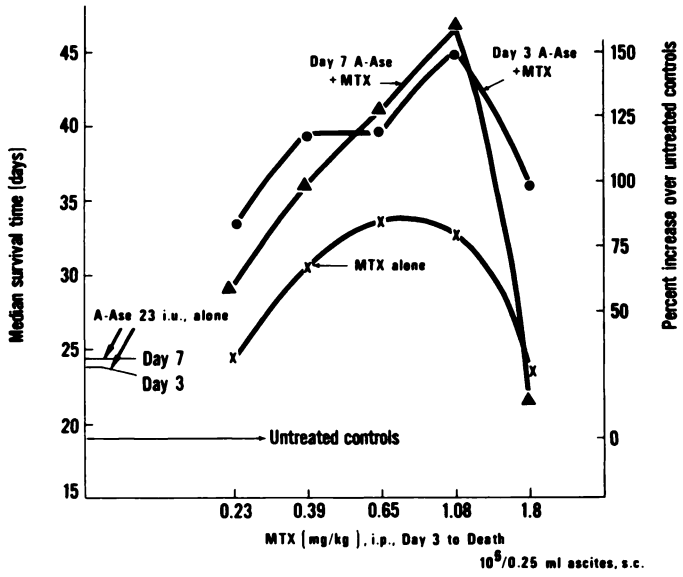

Chart 3. Influence of daily treatment with MTX starting before or after A-Ase injection on the survival time of leukemic mice; 8 treated mice/group and 20 untreated controls.

Daily Treatment with MTX, Starting 4 Days (Day 3) Prior to Single Treatment with A-Ase on Day 7. In the 2nd part of the experiment a single i.p. treatment with A-Ase (23 i.u.) on Day 7 at 9 a.m. increased the survival time of mice to 24.5 days (ILS, 36%). Similarly, when leukemic mice were treated daily from Day 3 with MTX, 0.23 to 1.8 mg/kg, and a single injection of A-Ase on Day 7, MST's of 22 to 46.5 days were obtained. Again, the most effective combination (MST, 46.5 days) was found to be A-Ase plus MTX, 1.08 mg/kg. Here, also, local tumor growth of leukemic mice treated with the combination of drugs appeared delayed as compared with tumor growth in mice treated with single drugs.

Schedule Dependency of Sequential Treatment with A-Ase and MTX

Another experiment was conducted to observe the effect of the sequence of daily treatments with A-Ase and MTX (Chart 4). In this experiment, the MST of the untreated control mice was 15 days, and for those treated with A-Ase (Days 3 to 7) or MTX (Days 10 to 14) alone the MST's were 19 and 19.5 days, respectively. No therapeutic synergism was observed in the mice treated with A-Ase from Days 3 to 7 followed by MTX from Days 10 to 14 (MST, 16 days). In contrast, therapeutic synergism was observed in mice treated with MTX (3 mg/kg) from Days 3 to 7 followed by A-Ase (23 i.u.) from Days 10 to 14. The MST of mice treated with MTX or A-Ase alone were 21 and 19.5 days, respectively, and in the mice treated with the combination (MTX followed by A-Ase) the MST was 32 days (ILS, 118%).

Thus, 5 treatments of A-Ase followed by 5 treatments of MTX were not therapeutically effective; in contrast, marked therapeutic synergism was observed when 5 treatments of MTX were followed by 5 treatments of A-Ase.

Effect of Treatment with MTX on the Survival Time of Leukemic Mice Bearing L5178Y or Its A-Ase Variant Line

Since treatment with A-Ase (Days 3 to 7) prior to MTX (Days 10 to 14) may have antagonized the therapeutic ac-

tivity of MTX, an experiment was conducted to study whether tumor cell resistance to A-Ase would influence the therapeutic activity of MTX. Mice were given s.c. injections of either L5178Y or its A-Ase-resistant variant line and treated i.p. with MTX. The results are summarized in Table 2. The MST of the untreated control mice bearing L5178Y-sensitive or A-Ase-resistant tumor was 16 days. A single i.p. treatment with A-Ase (23 i.u.) in mice bearing the sensitive tumor increased the MST from 16 to 24.5 days, whereas the MST of mice bearing resistant tumor was similar to that of the untreated controls. The MST (range) of mice bearing sensitive tumor were 7 to 19.5 days and those bearing resistant tumor were 7.5 to 20 days when treated on Day 3, with a single injection of MTX, 39 to 300 mg/kg. In the leukemic mice treated with MTX (9 to 23 mg/kg) every 4 days from Day 3 the MST range were 15.5 to 27 days in mice with sensitive tumor and 15 to 24.5 days in mice with resistant tumor. When daily treatment from Day 3 with MTX (0.39 to 3.0 mg/kg) was given, the MST range of mice bearing sensitive tumor were 12.5 to 24 days and that for those bearing resistant tumor were 11.5 to 27.5 days. In general, it appears that the tumors, sensitive and resistant to A-Ase, were equally sensitive to MTX treatment, indicating that tumor resistance had no influence on the reduction of therapeutic effectiveness when daily A-Ase was followed by daily MTX.

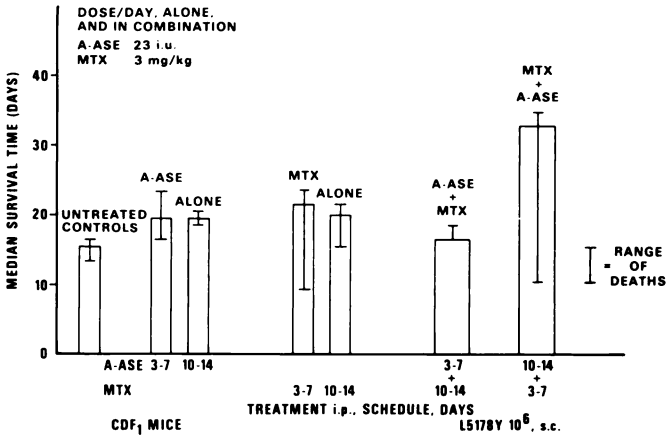

Chart. 4. Schedule dependency of combination treatment with A-Ase and MTX in leukemic mice; 8 treated mice/group and 20 untreated controls.

DISCUSSION

These studies indicate that the combination of a single dose of A-Ase with daily MTX is synergistic over a variety of schedules in the treatment of leukemia L5178Y. Five daily treatments with MTX followed in 3 days by 5 daily treatments with A-Ase also proved to be markedly better than a single course of either agent alone.

Capizzi *et al.* (5) observed that when L5178Y leukemic mice were treated with MTX on Days 3, 7, and 11 and A-Ase on Days 4, 8, and 12 there were 90-day survivors, whereas the converse regimen was less effective than MTX alone. Connors and Jones (6) showed that, in CBA mice bearing R_1 lymphoma, combination of A-Ase and MTX given simultaneously were significantly less effective than when a course of MTX was followed in 3 days by a course of A-Ase or *vice versa*. Similar results were reported by Mashburn (11) in experiments with lymphosarcoma P1798 in BALB/c. mice. Using a variety of antitumor agents in combination with A-Ase, it was shown that simultaneous treatment with A-Ase and other drugs failed to show therapeutic advantage, but when A-Ase was given as

Table 2

Effect of treatment with MTX on the survival time of mice[a] bearing L5178Y leukemia or on the subline resistant to A-Ase[b]

Schedule of treatment i.p.	Drug (mg/kg)	Dose (mg/kg)	L5178Y sensitive to A-Ase MST[c] (days)	Range (days)	% ILS[d]	L5178Y resistant to A-Ase MST[c] (days)	Range (days)	% ILS[d]
Untreated controls			16	11-20		16	13-19	
Single treatment on Day 3 only	MTX	39	19.5	17-21	22	18.5	8-23	15
		65	19	7-21	19	20	10-23	25
		108	19	7-21	19	9	5-10	
		180	8	7-18		7.5	7-9	
		300	7	7-10		7.5	7-9	
Every 4 days from Day 3 to death	MTX	9	20	20-21	25	19.5	11-28	22
		14	27	19-29	68	24.5	18-28	53
		23	15.5	10-21		15	10-25	
Daily from Day 3 to death	MTX	0.39	22	20-23	37	27.5	26-28	71
		0.65	24	20-26	50	24	18-29	50
		1.08	18.5	15-25	15	20.5	10-29	28
		1.8	16	13-17		11.5	10-25	
		3.0	12.5	10-14		13	10-15	
Single treatment on Day 3 only	A-Ase	23 i.u.	24.5	22-27	53	14	9-17	

[a] $CD2F_1$ mice were inoculated s.c. with 10^6 cells sensitive or resistant to A-Ase.

[b] $CD2F_1$ mice bearing L5178Y ascites treated on Day 3 with A-Ase (23 i.u.), the resistant line maintained in mice by serial passages and treatment at every generation.

[c] Of 8 mice/group.

[d] ILS of MST over untreated controls.

a primary agent followed by a single drug or combination of drugs therapeutic synergism and cures were obtained. This report also showed antagonism between MTX and A-Ase following simultaneous administration although the degree of antagonism was related to the stoichiometric ratio of the 2 agents.

The schedule-dependent antagonism between MTX and A-Ase seen in these tumor systems is probably due to antagonism of the unbalanced growth mechanism of MTX cytotoxicity (1) by concurrent inhibition of protein synthesis by A-Ase as shown in the *in vivo* and *in vitro* studies of this combination in L5178Y leukemia by Capizzi *et al.* (4). The drug schedules used in the present study in which a single dose of A-Ase was combined with daily MTX would minimize this type of biochemical interaction. The 1 schedule where therapeutic synergism was not observed, 5 daily treatments of A-Ase followed 3 days later by 5 daily treatments of MTX, could be due to antagonism of the action of MTX by some residual effects of A-Ase. It is also possible that the 3-day interval between A-Ase and MTX permitted recovery of the leukemic cell population to an extent where MTX was ineffective. It is known that the therapeutic activity of MTX decreases with increased tumor size and the subsequent decreased growth fraction (9). Connors and Jones (6) found that this schedule was synergistic in the treatment of i.p. R_1 lymphoma. The inherent differences in tumor response or growth rate may be a basis for this discrepancy. The basis for failure of this sequential schedule in these experiments would not appear to be attributable to host toxicity alone as was observed when A-Ase treatment was preceded by cyclophosphamide (17) since the mice receiving combination treatment of A-Ase plus MTX began to die after the untreated controls. It can be inferred from the data in Table 2 that this sequential schedule did not fail because of the appearance of A-Ase-resistant tumor cells as these cells are as sensitive to MTX as the A-Ase-sensitive line.

Riley *et al.* (14) reported that the half-life of A-Ase and its chemotherapeutic effectiveness in transplantable tumors are increased in the presence of LDH-elevating virus. Our mouse sera samples contained high LDH activity ranging from 11,400 to 11,800 units in normal DBA/2 mice, 5,800 to 7,300 in normal $CD2F_1$ mice, and 14,000 to 26,200 units in DBA/2 mice bearing the L5178Y tumor. Thus, it would appear that both the mice and the tumor carry the LDH virus. It is not clear to what extent the presence of LDH virus may have influenced the activity of A-Ase alone or A-Ase in combination with MTX in these studies. It is possible that a longer half-life for A-Ase in the presence of the virus may have contributed to the therapeutic synergism observed. Yet it is not clear how an increase in A-Asc half-life could have failed to yield therapeutic synergism in the instance in which 5 treatments with A-Ase were followed 3 days later with 5 treatments of MTX; whereas, on the reciprocal schedule, with MTX administered first, therapeutic synergism was observed. In any event, determination of therapeutic synergism of A-Ase in combination chemotherapy of L5178Y leukemia in a milieu devoid of LDH virus is complicated by the ubiquitous nature of the virus in the tumor cells and mouse colony.

There is evidence in the leukemia L1210 system that the therapeutic synergism can be obtained with combinations of MTX with several antitumor agents (8, 9). Similarly, combinations of established cytotoxic drugs with A-Ase have proven synergistic in asparagine-requiring tumor systems (3, 11). These studies indicate the need for developing proper schedules for combinations of A-Ase and MTX to increase the therapeutic efficacy of these drugs.

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