The Effect of Supralethal Amethopterin and Folinic Acid Rescue on Mouse Skin Allograft Survival[1] (35300)

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Amethopterin has been considered to be a relatively ineffective immunosuppressive drug in mice as there have been no reports of significant prolongation of skin grafts involving H-2 histocompatibility differences. This is probably due to the fact that mice seem to be extremely sensitive to the toxic effects of this agent, and thus the therapeutic index is very low. Nevertheless, some therapeutic effect has been demonstrable in specific experimental systems such as the suppression of the graft-versus-host reactions which occur in irradiation chimeras (1), neonatal runt disease (2), and parabiotic intoxication (3). Since amethopterin as a folic acid antagonist has a natural occurring "antidote," folinic acid, it presents considerable theoretic appeal at the clinical level where immunosuppression is potentially lethal. Berenbaum and Brown (4) have applied this antidote principle in successfully suppressing the humoral antibody reponse to typhoid antigen in mice by administering a supralethal dose of amethopterin and then "rescuing" the animals with folinic acid a few hours later. In the present experiments this principle was used to suppress the responses to skin grafts involving major histocompatibility differences.

Materials and Methods. Groups of 6-week-old A/Jax mice (H-2^a) served as the test animals and received varying doses of amethopterin subcutaneously according to the method of Berenbaum and Brown (4). Dosage levels of amethopterin of 15, 30, 60, and 100 mg/kg were employed on an every other day, every 5 day and every 7 day basis. Folinic acid, 200 mg/kg, was given intraperitoneally as a "rescuing agent." The postinjection-rescue interval was varied from 6 to 18 hr to determine the optimum regimen. Toxicity was measured by loss of body weight and by mortality rate. Skin grafts from C57BL/6 (H-2^b) were applied according to the method of Billingham and Medawar (5); and skin graft survival was recorded as median survival time (MST $\pm$ 1 SE) as described by Monaco *et al.* (6). Both pregraft and postgraft treatment were evaluated. The effect of amethopterin has been likened to that of irradiation. Since adult thymectomy has been demonstrated to prolong the immunosuppression induced by irradiation (7) groups of A/Jax mice had transcervical aspiration thymectomy performed before treatment in an attempt to enhance further the suppressive effect of amethopterin plus rescue.

Results. a. Drug toxicity studies. Preliminary studies confirmed that female mice seemed to be more sensitive to amethopterin than males, and therefore male mice were used thereafter. Mice given a single dose of amethopterin of up to 100 mg/kg, followed by folinic acid rescue 8 hr later, suffered no mortality, a finding which agrees with that of Berenbaum and Brown (4). Thus, folinic acid rescue allows mice to tolerate up to four times the LD_{50} of a single dose of amethopterin (about 25 mg/kg). However, when a multiple-dose schedule was employed, significant toxicity and mortality was observed. Dose–response curves are difficult to obtain because of significant variation in response. Uniform results, however, were obtained by repeating each experiment several times utilizing a fixed dose of drug and varying the administration intervals in contrast to when the dose itself was varied. In the former instance although one dose of 70 mg or 100 mg/kg with rescue was well tolerated, re-

[1] This work supported in part by Grants AI-06320 and AM-07055 from the United States Public Health Service.

TABLE I. The Effect of Folinic Acid Rescue on Mortality After Amethopterin (30 mg/kg).

Dose frequency (days)	Mortality (%)			
		Rescue after (hr):		
	No rescue	18	8	6
Once	72	0	0	0
Every 7[b]	85	33	18	0
Every 5[b]	100	55	42	17
Every 2[b]	100	78	55	50

[a] 200 mg/kg.
[b] Given for 4 weeks.

peated administration of such doses on any schedule was invariably lethal, obscuring any margin of benefit from the folinic acid rescue. However, with a dose of 30 mg/kg, the addition of folinic acid rescue was associated with considerable improvement in survival at all frequencies of administration as shown in Table I. When there was greater than a 7-day interval between amethopterin injections, the timing of folinic acid rescue was not critical within a 24-hr period. An increased frequency of administration, however, did demonstrate the importance of the time interval between amethopterin and rescue (also shown in Table I) with an inverse relationship between increasing dose frequency and decreasing rescue interval. Moreover, at each dose frequency the mortality was least with the shortest rescue interval.

b. Skin graft survival. Amethopterin pretreatment in varying dosage was investigated and alone was totally without effect in prolonging skin graft survival. Increased toxicity and mortality without improved results were also observed in mice which had combined pre- and postgraft treatment over those with postgraft treatment alone. These findings were not further investigated and a prolonged period of pretreatment was abandoned in favor of beginning therapy the day before the skin graft was applied. Several treatment regimens were tested on a postgraft basis against the C57BL/6 to A/Jax skin allograft response. Median survival time (MST ± SE) in this strain combination for normal first set rejection is 9.8 ± 0.2 days. A 30 mg/kg dose with folinic acid rescue 6 hr later given every 5 days gave the best results from the standpoint of survival as well as therapeutic effect as measured by a skin graft survival of 18.4 ± 2.3 days as shown in Table II. Table III suggests that some mortality and morbidity must be expected (20% in this group) for in experiments where minimal mortality occurred only slight graft prolongation was observed. Interestingly, similar mortality rates did not necessarily correlate with skin allograft prolongation and the group with the greatest mortality (group 6) did not manifest the greatest graft survival, which implies other variables. However, further evidence that some toxicity must be suffered to achieve the desired result is the fact that all of the surviving animals with prolonged skin grafts showed toxic effects of amethopterin, *i.e.*, diarrhea, hunched back, ruffled coat, and a 15–20% weight loss. What has not been evident before, however, is that folinic acid rescue has uncovered a narrow range of therapeutic effectiveness without increased toxicity since an 8-day skin graft prolongation was achieved with the 20% mortality range (group 3 in Table III).

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TABLE II. The Effect of Amethopterin and Rescue with and without Thymectomy in Skin Allograft Survival.

Group	Treatment	No. of animals	Mortality (%)	MST ± 1 SE (days)	No. of grafts surviving (days)					
					10	10–13	14–16	17–19	20–22	>22
1	None	20	0	9.8 ± 0.2	11	9	0	0	0	0
2	Amethopterin[a]	40	20	18.4 ± 2.3	0	6	6	10	5	4
3	Thymectomy	15	0	10.1 ± 0.8	9	6	0	0	0	0
4	Amethopterin[a] and thymectomy	34	0	13.2 ± 1.1	0	16	12	4	2	0

[a] Amethopterin (30 mg/kg) given every 5 days with folinic acid rescue (200 mg/kg) 6 hr later.

TABLE III. The Effect of Amethopterin and Rescue on Weight Loss, Mortality, and Skin Graft Survival.

Group	Dose (mg/kg)	Frequency interval (days)	Rescue[a] after (hr)	Wt loss (%)	Mortality (%)	Graft survival (days)
1	15	2	8	14	15	13
2	15	7	8	13	11	12
3	30	5	6	20	18	18
4	30	7	6	7	0	11
5	60	5	6	24	31	14
6	60	7	8	21	78	15

[a] 200 mg/kg of folinic acid.

previous thymectomy upon amethopterin treatment was surprising since thymectomized mice exhibited an increased resistance to the drug. The animals lost less weight than intact controls on identical treatment schedules, and this was noted in both grafted and ungrafted animals. On the most effective immunosuppressive regimen (30 mg/kg; 6-hr rescue; every 5 days) mice with previous thymectomy only lost 10–12% of their body weight as compared to 17–20% in intact mice. Table II shows the effect was also exhibited in the mortality rates where the 0% mortality in thymectomized recipients was in sharp contrast to the 18% mortality in similarly treated intact recipients. Similar differences were noted in other dosage regimens. The thymectomy effect was further manifested by a noticeable lessening of skin graft prolongation as shown in Table II where the median survival time in thymectomized–amethopterin-treated recipients was shortened to 13.2 $\pm$ 1.1 days. A nonspecific effect of operation was ruled out by equivalent results in animals which had thymectomy performed 4 weeks before drug treatment and animals with thymectomy only 3 days before. The effect was not observed in sham-operated and splenectomized animals, which result indicates thymus specificity.

Discussion. These experiments tend to contradict previous evidence that amethopterin is not an effective immunosuppressive drug for adult mice and lend further support to the principle of folinic acid protection. The most difficult test system possible using (a) a strain of mice which is very susceptible to amethopterin toxicity, and (b) skin graft survival involving major histocompatibility differences, was chosen purposefully to make sure the method had validity. The folinic acid protection principle has already been demonstrated to be effective clinically in patients with malignant head and neck tumors who have received intra-arterial perfusions of large doses of amethopterin and simultaneous systemic protection with intramuscular folinic acid (8). This report, however, is evidence that such a method may be useful in the abrogation of the transplantation rejection reaction since it is clear that the very small margin which exists between ineffective and lethal doses of amethopterin may be broadened by using a delayed rescue of folinic acid.

Several interesting biological phenomena bear reemphasis. First, male mice are definitely less sensitive than females to amethopterin, not only given alone but also in the supralethal doses plus rescue regimen used here. Why this may be has not been explained. Second, pretreatment, in this form of drug induced immunosuppression, was without effect alone, and without additional effect if used in animals also receiving the drug after grafting. This is just one additional bit of evidence in favor of those theories claiming that there are no benefits from pregraft treatment with immunosuppressive cancer chemotherapeutic and related agents. Actually this only seems logical in light of the known mechanisms of action of amethopterin, which is a potent inhibitor of folic acid reductase, an essential enzyme in thymidine synthesis (9). Since the margin is small, the therapeutic effect may not be seen in a pre-

grafting regimen where the drug is directed against a "resting" immune system, the cells of which should be equally susceptible to the inhibitors of nucleic acid synthesis. However, in a stimulated system where immunologically competent cells are activated by graft antigen and dividing, such cells may be selectively affected by the drug which then is measurable as an immunosuppressive effect (10).

A third point is additional confirmation of Uphoff's contention that some toxicity must be suffered in order to obtain the desired effect (1). In these experiments, 15–20% mortality was necessary to obtain significant graft prolongation and all survivors suffered toxic manifestations. It might be anticipated that even more striking results might be obtained using a donor–recipient combination eliciting a weaker transplanation response, or one in which the immune mechanisms have already been partially subdued by other immunosuppressive measures. It was with this in mind that the experiments involving thymectomy were undertaken with the resulting unexpected findings. Why the absence of thymus "protects" mice from the effects of amethopterin is totally unexplained. But since the protection against toxicity also diminishes the immunosuppressive effect, it is further indication that suppression and toxicity are directly related. It would be tempting to speculate that the suppressive effects of amethopterin may be mediated through the thymus and that in its absence the possibly more amethopterin-resistant peripheral lymphoid centers and cells carry on the allograft reaction unabated by the presence of the drug.

Summary. The folic acid antagonist amethopterin has been utilized as an immunosuppressive agent in mice in supralethal dosages combined with delayed administration of folinic acid as a rescuing agent. This principle has been found effective in significantly prolonging the survival of skin grafts involving major histocompatibility differences. A schedule of injections of 30 mg/kg body weight given subcutaneously, with a 200 mg/kg folinic acid rescue intraperitoneally 6 hr later, given every 5 days, allowed maximum graft survival of up to 18 days. Pregraft treatment was found to be without effect, and previous thymectomy seemed to exert a "protective" effect against both the therapeutic and toxic manifestations of amethopterin therapy.

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Received Oct. 7, 1970. P.S.E.B.M., 1971, Vol. 136.