Enhanced Suppression of Experimental Allergic Encephalomyelitis by Combination Chemotherapy with Duazomycin-A and 6-Mercaptopurine* (33852)

CHARLES L. VOGEL[1] AND PAUL CALABRESI
(Introduced by A. D. Welch)
Yale University School of Medicine, New Haven, Connecticut 06520

Experimental allergic encephalomyelitis (EAE) is a demyelinating disease that is widely held to be the result of a delayed hypersensitivity reaction of an "autoimmune" type. The discovery of effective therapy for this disease could have important clinical implications for human diseases of hypersensitivity as well as for the field or organ transplantation. Hoy *et al.* (1) were the first to use thiopurines in the treatment of EAE. Since that time several other antineoplastic drugs have proved effective in transiently suppressing clinical and histologic evidence of disease during the period of drug administration (2–5). With all these agents, however, it is usual for manifestations of EAE to develop after discontinuation of therapy.

The use of combination chemotherapy of EAE in the present study was suggested in part by successes with drug combinations in the therapy of some neoplastic diseases (6) and by reports of the enhancement of theiopurine activity by glutamine antagonists in general (7, 8) and by Duazomycin-A (*N*-acetyl-6-diazo-5-oxo-L-norleucine) (DZM), in particular (9, 10).

In the present study, attempts were made to suppress EAE by treating with 6-mercaptopurine (6-MP) and DZM alone and in combination. The data indicate that DZM enhances the effectiveness of 6-MP in EAE. A preliminary report of these findings has been presented (11).

Materials and Methods. An encephalitogenic emulsion was prepared by adding a suspension of dry, killed *Mycobacterium tuberculosis* (4 mg/ml) in Bayol F mineral oil to freshly ground whole guinea pig spinal cord. The concentration of spinal cord in the completed emulsion was 550 mg/ml (wet wt). Wistar rats (100–150 g) were sensitized with a single 0.2-ml dose of encephalitogenic emulsion injected intradermally into a hind foot pad. Therapy in all groups was initiated at the time of challenge and continued through day 16. A 6-MP[3] solution was prepared by dissolving the powdered drug in 1 *N* NaOH and adjusting the pH to 10 by appropriate dilution with isotonic saline; this was administered intraperitoneally. DZM[4], obtained in concentrations of 40 mg/ml and diluted to 0.01 mg/ml with isotonic saline, was administered subcutaneously. The control group received 1 ml of a dilute NaOH solution at a pH 10 intraperitoneally.

Before assignment to the various experimental groups, all animals were observed for at least 1 week for signs of spontaneous central nervous system (CNS) disease. After challenge with encephalitogenic emulsion, the animals were weighed and observed daily for signs of EAE. Parameters recorded included weight loss, fecal impaction, tremor, and difficulty in righting. For the purpose of the study, however, an animal was not considered to have EAE unless unequivocal hind limb weakness developed. Weakness was used as

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[1] Clinical Associate, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.

[2] Burroughs Wellcome Scholar in Clinical Pharmacology. Present address: Brown University, Department of Medicine, Roger Williams General Hospital, 825 Chalkstone Avenue, Providence, Rhode Island 02908.

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TABLE I. Results of Drug Therapy in Experimental Allergic Encephalomyelitis.

Group	Therapy (mg/kg)	No. of rats	No. of rats with clinical disease		% with disease
			Before day 15	After day 15	before day 15
I	Buffer (no drug)	12	10	0	83
II	DZM (0.1)	6	6	0	100
III	6-MP (6)	10	7	2	70
	(12)	15	8	5	53
	(25)	10	5	4	50
	(50)	6	4	2	67
IV	DZM (0.1) + 6-MP (6)	22	11	8	50
	DZM (0.1) + 6-MP (12)	12	2	6	16

the end point in Tables I and II, rather than paralysis, since many animals were sacrificed soon after onset of EAE for clinicopathologic correlation. In addition, spontaneous recovery from EAE is frequent in Wistar rats and many animals never progress beyond a stage of mild weakness. The discovery that the Lewis strain of rats is more susceptible to EAE than other strains was reported after the completion of these studies (12). It is anticipated that we shall employ the Lewis strain in future attempts at chemotherapeutic suppression of EAE.

For the purpose of establishing a clinicopathologic correlation, at least half of the animals in each group (except for the group given only 6-MP in daily doses of 50 mg/kg) were sacrificed at varying intervals after day 13; and histologic sections of brain and spinal cord were prepared and stained with hematoxylin and eosin. Additional sections were stained with Weil's myelin stain. Histologic lesions were graded as follows: O = no pathology; mild = occasional discrete perivasular infiltrates; and severe = marked perivascular inflammation, easily seen in almost every field.

Observations of clinical signs and the degree of histologic involvement with EAE were made and recorded independently. In Table III, animals not progressing from a stage of hind limb weakness to paralysis were consid-

TABLE II. Comparison of Effects of 6-MP and Combinations of 6-MP and DZM on Day of Onset EAE.

Treatment schedule:	Control	6-MP (6 mg/kg)	6-MP (6 mg/kg) + DZM (0.1 mg/kg)	6-MP (12 mg/kg)	6-MP (12 mg/kg) + DZM (0.1 mg/kg)
No. of animals:	11	9	22	14	11
Day of onset EAE					
10	++				
11	+	++		+	
12	++++	+++	++	+++	
13	++	+	++++	++	
14		+	++	+	+
15			+++	+	+
16	+		+		
17				+	+
18		+	+++	++	+
19		+			++
>20	+		+++++++	+++	+++++

[a] Each plus represents day of onset of EAE in one animal.

TABLE III. Correlation (no. of animals) of Clinical Observations and Histologic Observations in 47 Rats Given Whole Guinea Pig Cord in Adjuvant and Treated with Various Chemotherapeutic Regimens.

	Histology		
Clinical stage	Negative	Mild	Severe
Unaffected	4	6	0
Mild	1	3	12
Severe	1	1	19
Total	6	10	31

ered as having clinically "mild" disease, whereas paralyzed animals were considered "severe."

Results, clinical. Table I summarizes the clinical results of all experimental groups while Table II presents further analysis of the significant results (statistical evaluation of data presented in Table II was performed by rank analysis). Total numbers of animals in Table I are greater than those in Table II since all animals clinically free of disease that were sacrificed before day 20 are ommitted from Table II.

Duazomycin-A at a dose 0.1 mg/kg afforded no protection against EAE when used alone (Table I). The 6-MP (12 mg/kg) significantly delayed the onset of clinical disease when compared with controls ($p<.05$, Table II). Animals not developing EAE during 6-MP therapy usually developed the disease from 1 to 9 days after the drug was discontinued. The inability of 6-MP to afford permanent protection from EAE is evident when all 6-MP treated animals regardless of dose are considered together. Thirty-seven of 41 (90%) 6-MP-treated animals ultimately developed EAE. There was no difference in the degree of severity or rate of progression of EAE developing after discontinuation of 6-MP and EAE observed in control animals.

From Table II it is evident that the day of onset of EAE in animals receiving 6-MP daily (6 mg/kg) alone was earlier than in those animals treated with the same daily dose of 6-MP plus DZM (0.1 mg/kg) ($p<.02$). Enhancement of 6-MP action by DZM is also evident in animals given 6-MP in daily doses of 12 mg/kg. At this dose, animals treated with 6-MP alone had a significantly earlier day of onset than those treated with the combination ($p<.05$). It is of particular interest that 4 of 12 animals (33%) treated with this latter combination never developed clinical signs of disease even after discontinuation of therapy.

Drug toxicity. There were no deaths that could be attributed to drug toxicity in any experimental group. Since total leukocyte and platelet counts were not performed in this study, precise evaluation of subclinical degrees of bone marrow suppression was not possible. All animals either died of progressive EAE or were sacrificed for histologic study but none had any obvious infectious complication or hemorrhagic manifestations. Weight loss in treated animals was not significantly different from controls and was usually better correlated with the severity of EAE rather than on the basis of drug toxicity.

Clinicopathologic correlation. There was no demonstrable histological difference between the brains of control animals with severe CNS lesions and those of treated animals. As noted in Table II, there was good clinicopathologic correlation between clinical manifestations and histologic staging of disease in 26 of 47 animals. In 18 of 47 rats, the histologic lesions were more extensive than suggested by clinical stage, while in only 3 animals were the histologic lesions less extensive than suggested by clinical evaluation. Two of these 3 animals had clinical disease and no histologic evidence of EAE in the sections studied, but serial sections were not performed.

Discussion. Many agents used in the chemotherapy of neoplastic diseases have suppressed immune responses in animals and in man (13). Knowledge of the immunosuppressive effects of these agents has been exploited in the field of organ transplantation and in the therapy of so-called "auto-immune" diseases. Because of these practical considerations, many workers are directing their attention to finding more effective immunosuppressive chemotherapeutic regimens. Toward

this end, some investigators have used EAE as an experimental model of a hypersensitivity state and have tried to modify its course with different classes of drugs (5). Although considerable work has been performed in the treatment of EAE with individual agents, to date, little attention has been paid to the use of combination chemotherapy in this experimental disease. Our data indicate that the use of rational combination chemotherapy with 6-MP and DZM is more effective in EAE than therapy with either agent used alone. Further trials of such combinations in EAE and other experimental immunopathological disorders may offer additional experimental justification for cautious therapeutic trials in clinical hypersensitivity states (14, 15).

6-Mercaptopurine, a purine analog that has proved valuable in the treatment of leukemia, is capable of inhibiting both the primary and secondary immune responses to antigens that elicit humoral antibody (16), suppressing the allograft response (17), prolonging the survival of skin homografts in experimental animals (18), and improving the clinical status of some patients with clinical diseases associated with hypersensitivity (19). Bone marrow suppression is the primary toxic effect of 6-MP in both animals and man. DZM, an antibiotic isolated from *Streptomyces ambofaciens*, has activity against certain animal tumors (20, 21) and possibly human bronchogeniccarcinoma (22). When used alone, it was unable to suppress the humoral secondary immune response to diphtheria toxoid in rabbits (10). Its toxic effects involve the alimentary tract and the bone marrow.

Hoyer *et al.* (1) found that 6-MP in daily doses of 6–9 mg/kg given for 18 days starting at the time of encephalitogenesis could delay the average day of onset and decrease the severity of EAE in rabbits (1). They found that a daily dose of 12 mg/kg, although toxic, was still more effective in suppressing signs of EAE during the period of drug administration. Typical EAE did develop, however, after discontinuation of 6-MP therapy. In the present study, 6-MP modified the clinical manifestations of EAE in another species, the rat. In animals receiving 6-MP (12 mg/kg), the average day of onset was significantly delayed when compared with controls ($p = 0.05$). The differences between our study and that of Hoyer *et al.* probably reflect species differences in the response to, or the metabolism of, 6-MP; thus, in daily doses of 12 mg/kg, 6-MP was toxic to the rabbit and abolished all signs of EAE during drug administration, whereas daily doses up to 50 mg/kg for 15 days were nontoxic in the present study and complete suppression of clinical disease in all animals was not achieved. Other workers have also noted the relative resistance of rats to the toxic effects of 6-MP (23).

The major purpose of this study was to examine the use of a rational combination of chemotherapeutic agents in an experimental "auto-immune" disease. Although apparently ineffective when used alone, DZM enchanced the effect of 6-MP in suppressing EAE. This finding is consistent with other reports of enhanced activity achieved by combinations of thiopurines and glutamine antagonists. Combinations of these two classes of drugs have also proved active in experimental animal tumor systems (7, 8) and in suppression of the secondary humoral immune response to diphtheria toxoid (10). Limited clinical trials involving combinations of thiopurine and glutamine antagonists have already been undertaken in human neoplastic (9) "auto-immune" disorders (14) and renal transplantation (15). The good results obtained with the combination of 6-MP and DZM in the doses used in the present study were accomplished with apparent increase in toxicity.

The enhanced effectiveness of combination chemotherapy with 6-MP and DZM is thought to be due to multi-site blockade of sequential steps in the *de novo* pathway of purine biosynthesis. As illustrated in Fig. 1, DZM, a glutamine antagonist similar to *O*-diazoacetryl-L-serine (azaserine) and to 6-diazo-5-oxo-L-norleucine (DON), inhibits the formation of (α-*N*-formyl) glycineamidine ribonucleotide (FIGR) from (α-*N*-formyl)

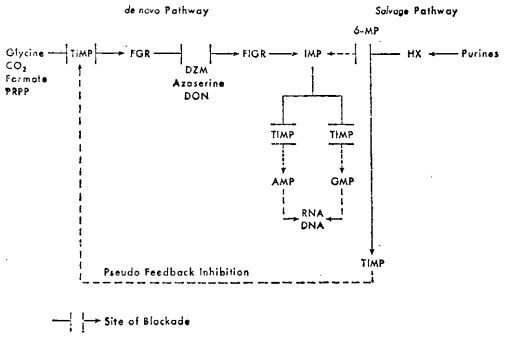

FIG. 1. Pathways of purine biosynthesis demonstrating probably sites of inhibition by 6-mercaptopurine (6-MP) and glutamine antagonists (DZM = Duazomycin-A).

glycineamide ribonucleotide and glutamine (7). On the other hand, 6-MP has been shown to have many possible sites of action, the three most significant of which are illustrated in Fig. 1. 6-Mercaptopurine competes with hypoxanthine (HX) (formed in the *salvage* pathway of purine synthesis) for inosinic acid pyrophosphorylase and is itself converted to thioinosinic acid (TIMP). In turn, TIMP inhibits the conversion of inosinic acid (IMP) to guanylic acid (GMP) and adenylic acid (AMP). TIMP, by a process termed "pseudo feedback inhibition," also interferes with the first step of the *de novo* pathway of purine biosynthesis. A recent detailed review describes the mechanisms of these and other sites of blockade by 6-MP (24).

It is hoped that the encouraging results with this rational form of combination chemotherapy will lead to further studies of the immunosuppressive effects of drug combinations. Experience with experimental hypersensitivity states such as EAE may prove useful by suggesting potentially useful immunosuppressive regimens for use in human disorders of altered immune reactivity and in organ transplantation.

Summary. Experimental allergic encephalomelitis (EAE) in rats was used to study the immunosuppressive properties of 6-mercaptopurine (6-MP) and a glutamine antagonist, Duazomycin A (DZM), alone and in combination. The results indicate that while DZM alone had no immunosuppressive activity, it significantly enhanced the effectiveness of 6-MP in this disorder without increasing toxicity. The favorable results with this rational combination of antimetabolities in an experimental "auto-immune" disease associated with delayed hypersensitivity suggests similar applicability to disorders characterized by altered immune reactivity in man.

1. Hoyer, L. W., Condie, R. M., and Good, R. A., Proc. Soc. Exptl. Biol. Med. **103**, 205 (1960).

2. Georgi, V. E., Honegger, C. G., Rieder, H. P., and Wuthrich, R., Helv. Physiol. Pharmacol. Acta **21**, 381 (1963).

3. Brandriss, M. W., Smith, J. W., and Friedman, R. M., Ann. N. Y. Acad. Sci. **122**, 356 (1965).

4. Paterson, P. Y., Hanson, M. A., and Gerner, E. W., Proc. Soc. Exptl. Biol. Med. **124**, 928 (1967).

5. Rosenthale, M. E. and Nagra, C. L., Proc. Soc. Exptl. Biol. Med. **125**, 149 (1967).

6. Frei, E., III, DeVita, V. T., Moxley, J. H., III, and Carbone, P. P., Cancer Res. **26**, 1284 (1966).

7. Tarnowski, G. S. and Stock, C. C., Cancer Res. **17**, 1033 (1957).

8. LePage, G. A., Clin. Pharmacol. Therap. **2**, 121 (1961).

9. Lefkowitz, E. R., Creasey, W. A., Calabresi, P., and Sartorelli, A. C., Cancer Res. **25**, 1207 (1965).

10. Rosenberg, S. and Calabresi, P., Nature **199**, 1101 (1963).

11. Vogel, C. L. and Calabresi, P., Clin. Res. **12**, 239 (1964).

12. Levine, S. and Wenk, E. J., Ann. N. Y. Acad. Sci. **122**, 209 (1965).

13. Santos, G. W., Federation Proc. **26**, 907 (1967).

14. Kaplan, S. R., Hayslett, J. P., and Calabresi, P., New Engl. J. Med. **278**, 239 (1968).

15. Murray, J. E., Merrill, J. P., Harrison, J. H., Wilson, R. E., and Dammin, G. J., New Engl. J. Med. **268**, 1315 (1963).

16. LaPlante, E. S., Condie, R. M., and Good, R. A., J. Lab. Clin. Med. **59**, 542 (1962).

17. Calabresi, P., Proc. Intern. Cong. Chemotherapy, 5th, Vienna **1967**, 409.

18. Meeker, W. R., Condie, R. M., Weiner, D., Varco, R. L., and Good, R. A., Proc. Soc. Exptl. Biol. Med. **102**, 459 (1959).

19. Goodman, H. C., Wolff, S. M., Carpenter, R. R., Andersen, B. R., and Brandriss, M. W., Ann. Internal Med. **59**, 388 (1963).

20. Oleson, J. J., Reith, A. R., Thie, R. S., Mjos, K. J., and Calderella, L. A., Federation Proc. **19**, 394 (1960).

21. Anderson, E. P. and Brockman, K. W., Biochem. Pharmacol. **12**, 1335 (1963).

22. Colsky, J., Franzino, A., Jones, R., Jr., and Shnider, B., Proc. Am. Assoc. Cancer Res. **3**, 216 (1961).

23. Thomas, A. N., Morton, D. L., Crane, J. T., and Gardner, R. E., Proc. Soc. Exptl. Biol. Med. **107**, 70 (1961).

24. Elion, G. B., Federation Proc. **26**, 898 (1967).

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Hepatic Drug Metabolism in Ten Strains of Norway Rat Before and After Pretreatment with Phenobarbital (33853)

JOHN G. PAGE AND ELLIOTT S. VESELL

Section on Pharmacogenetics, Laboratory of Chemical Pharmacology, National Heart Institute, Bethesda, Maryland 20014

Differences among species and among strains of a given species in metabolism of certain drugs have been reported frequently (1–4) and it is established clearly that activities of drug-metabolizing enzymes differ not only among various species but also among certain strains of some species. However, strain differences in inducibility of hepatic microsomal drug-metabolizing enzymes have been reported in detail only once before (5). The results showed that in various strains of rabbits there existed large differences in the extent to which phenobarbital pretreatment elevated activities of drug-metabolizing enzymes (5). These results were so dramatic that the present study was undertaken to determine whether in a more commonly used laboratory species, *Rattus norvegicus*, there occurred similar large strain differences in the basal and induced levels of two mixed function oxygenases, aniline hydroxylase and ethylmorphine *N*-demethylase.

Materials and Methods. Ten strains of the species *R. norvegicus* were obtained mainly from the NIH animal production section; in addition, LE came from Blue Spruce Farms (Altmont, New York); LEW from Simonson Laboratories (Gilroy, California); W from Huntingdon Laboratories (Philadelphia, Pennsylvania); ACI, LEW, and F344 (Fischer) from Microbiological Associates (Walkersville, Maryland). Eight of the strains were inbred: ACI, Alb (Albany), Buf (Buffalo), F344 (Fischer), LE (Long Evans), LEW (Lewis), M520 (Marshall) and W (Wistar). OM (Osborne Mendel) and SD (Sprague-Dawley) were the two outbred strains employed. At the time that experiments were performed, animals weighed between 200 and 225 g, were approximately 2 months old and were maintained for at least 2 weeks in our animal quarters on water and Purina chow *ad libitum*. Wild Norwegian rats were trapped in a garbage dump in New Freedom, Pennsylvania and were used after only 1 day in the laboratory. Kangaroo rats are a wild strain of the species *Dipodomys merriami*. They are of a species distinct from *R. norvegicus* and were trapped in the deserts of southern California. They were kept in our laboratory under the conditions described above for only 4 days prior to use.

In initial experiments sodium phenobarbital was injected ip in a dose of 100 mg/kg every 24 hr for 5 days. Subsequent experiments on induction were performed after only 3 days because the results revealed that maximum elevations of aniline hydroxylase and ethylmorphine *N*-demethylase activities occurred after 3 days (Fig. 1). Kangaroo rats were much more sensitive to phenobarbital, and to prevent appreciable mortality the dose was reduced to 50 mg/kg every 24 hr for 3 days. Control animals received saline injections (0.8 ml., ip) on each of 3 days. Rats were sacrificed by decapitation. Livers were immediately removed, rinsed in saline, blotted dry, and weighed. A 1:4 homogenate was prepared with 1.15% KCl in 0.02 *M* Tris buffer, pH 7.4. The homogenate was centrifuged at 4° for 20 min at 9000*g*. Ethylmorphine *N*-demethylase (6) and aniline hy-