Biochemical Pharmacology, Vol. 27, pp. 1771–1774.

0006-2952/78/0701-1771$02.00/0

SHORT COMMUNICATIONS

Effects of a nonsteroidal anti-inflammatory agent and phenobarbital on hepatic microsomal mono-oxygenases in adjuvant disease in the rat*

(*Received* 17 *November* 1976; *accepted* 19 *October* 1977)

Subdermal injection of heat-killed mycobacterium dispersed in mineral oil induces a polyarthritic syndrome in the rat. This animal model has similarities to certain aspects of the human disease [1, 2] and is extensively utilized in the routine testing of potential anti-inflammatory and immunosuppressant agents [3, 4]. Reductions in the hepatic microsomal drug-metabolizing enzyme system or mono-oxygenases (HMDS) have been reported in adjuvant disease [5–10]. Impairment in HMDS resulted in a reduced capacity for oxidative drug metabolism, and subsequently could alter the efficacy, duration of action or toxicity of drugs in this animal model. Lower LD_{50} values for indomethacin and phenylbutazone were reported in rats with adjuvant disease [11]. Since an impairment in HMDS has not been adequately demonstrated in human rheumatoid disease, the pharmacological and metabolic profile derived from the adjuvant rat may or may not be representative of the therapeutic properties of the drug substance in man. However, the adverse effects of these drugs, which often limit their use, particularly in chronic therapy, could be associated with a decreased HMDS capacity in human rheumatoid disease.

Fenclorac (α,*m*-dichloro-*p*-cyclohexylphenylacetic acid, diethyl-ammonium salt) is a new nonsteroidal anti-inflammatory drug which is more active than phenylbutazone both in acute and chronic animal models of inflammation [12], and has less gastrointestinal toxicity than indomethacin [13]. In this study, the effect of fenclorac on HMDS in adjuvant disease was determined and correlated with the anti-inflammatory properties of this compound. Since phenobarbital, a known inducer of hepatic microsomal mono-oxygenases [14], has been demonstrated to elevate the impaired HMDS to nearly normal levels in rats either with developing or established adjuvant disease [8, 10], the effect of combined fenclorac and phenobarbital therapy on HMDS was also studied in this animal model.

Methods

Male Sprague–Dawley rats, weighing 182–217 g, were obtained from ARS/Sprague–Dawley, Madison, W1, and were housed in groups of three animals per cage. The animals were maintained on a standard laboratory diet. No hydrocarbons or saw dust were employed in the laboratories or the animal quarters. The drugs were administered *per os* every 24 hr, as designated in a dosing volume of 5 ml/kg.

Adjuvant disease was induced by subdermal injection of adjuvant [0.5 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, Mich.) in 0.1 ml of light mineral oil] into the distal portion of the tail. In experiments designed to study the role of phenobarbital in adjuvant disease, daily phenobarbital treatment began 3 days prior to adjuvant administration and continued up to day 15 (nineteen doses). Animals received fenclorac (3 mg/kg) with or without the co-administration of phenobarbital (75 mg/kg) daily from days 0 to 15 after adjuvant injection. The control animals received an equivalent volume of the vehicle (distilled water).

Paw volumes. The severity and progress of the adjuvant disease was measured by assessment of paw volumes by the mercury displacement method of Winter *et al.* [4] and measurements of plasma inflammation units [10]. The unit of measurement for paw volumes was calibrated so that 40 units were equivalent to 1 ml of mercury displacement. The volume displacement capacity of the instrument was 5 ml, or 200 units, and full-scale measurements were linear.

Preparation of microsomes. Liver homogenates (20%, w/v) were prepared from tissues of two to four rats in 0.1 M phosphate buffer (pH 7.4) by a modification of previously described methodology [15]. The liver homogenate was centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant fraction was centrifuged at 104,000 *g* for 60 min in a Beckman LS5-50 ultracentrifuge at 4°. The pellet (microsomal fraction) was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl in such a volume that each ml was equivalent to approximately 0.4 g of the original liver wet weight. The microsomes were washed once with 0.15 M KCl, prior to the preparation of microsomal suspensions in the determination of cytochromes.

Determination of hepatic drug-metabolizing enzymes activity. The activities of aminopyrine demethylase, aniline hydroxylase and NADPH–cytochrome *c* reductase, and the contents of cytochrome P-450, cytochrome b_5 and microsomal protein were measured by procedures reported earlier [16]. There were no significant differences in the levels of cytochrome P-450 and cytochrome b_5 or in the activities of NADPH–cytochrome *c* reductase, aminopyrine demethylase and aniline hydroxylase in the control groups of rats receiving distilled water *per os* or a subdermal injection of the adjuvant vehicle (light mineral oil).

Statistical calculations were performed according to Snedecor and Cochran [17] and values for the two-tail Student's *t*-test of $P < 0.05$ were considered significantly different.

Results and discussion

Fenclorac reduced the severity of polyarthritic syndrome as indicated by reductions in paw edema (82 per cent) and plasma inflammation units (42 per cent) in rats with developing adjuvant disease (Table 1), with only a marginal improvement in reductions in HMDS (Table 2). Although phenobarbital alone inhibited paw edema by 30 per cent, the combined treatment with fenclorac and phenobarbital reduced the paw edema by 81 per cent. Chronic administration of phenobarbital during the development of primary and secondary lesions prevented the

* This paper was presented at the 1976 Fall Meeting of American Society for Pharmacology and Experimental Therapeutics in New Orleans, LA, August 1976 [*Parmacologist* **18**, 218 (1976)].

Table 1. Effect of fenclorac and phenobarbital on paw edema and plasma inflammation units in rats with developing adjuvant disease*

Drug	Dose (mg/kg/day)	Paw volume units†		% Inhibition of paw edema‡	Plasma inflammation units
		Day 0	Day 14		
Controls		48.6 ± 0.6	77.7 ± 3.9		100 ± 11
Phenobarbital	75	48.2 ± 0.7	68.6 ± 4.4	30	90 ± 9
Fenclorac	3	51.5 ± 1.1	56.7 ± 1.6§	82§	58 ± 13§
Fenclorac + phenobarbital	3 + 75	51.4 ± 0.7	56.9 ± 2.8§	81§	63 ± 11§

* All drugs were administered orally daily for 14 days prior to the measurement of paw edema and plasma inflammation units. All values are the means ± S.E. from ten to twelve rats in each group.

† One ml paw edema = 40 units (see text for details).

‡ 100 $(x-y)/x$, where x = mean paw units (day 14–day 0) in adjuvant control groups, and y = mean paw units (day 14–day 0) of drug group.

§ $P < 0.05$, compared to controls.

Table 2. Effect of fenclorac and phenobarbital on hepatic microsomal mono-oxygenases in rats with adjuvant disease*

Parameter	Normal		Adjuvant			
	Control	Phenobarbital	Control	Phenobarbital	Fenclorac + phenobarbital	Fenclorac
Liver wet wt (g/100 g body wt)	3.04 ± 0.05	4.53 ± 0.14† (149)	4.05 ± 0.60 (133)	4.80 ± 0.09† (158)	4.89 ± 0.13†‡ (161)	3.72 ± 0.15† (122)
Microsomal proteins (mg/g)	33 ± 1	48 ± 4† (146)	31 ± 0.1†§ (94)	35 ± 1‡§ (106)	39 ± 2‡ (118)	32 ± 1§ (97)
Aminopyrine *N*-demethylase (nmoles 4-aminoantipyrine formed/mg/30 min)	7.8 ± 0.3	25.4 ± 3.3† (326)	1.8 ± 0.2†§ (23)	14.5 ± 1.8†‡§ (186)	13.1 ± 1.9‡§ (168)	2.1 ± 0.4†§ (27)
Aniline hydroxylase (nmoles *p*-aminophenol formed/mg/15 min)	9.1 ± 0.2	13.4 ± 0.9† (147)	3.8 ± 0.5†§ (42)	9.9 ± 2.0‡ (109)	9.6 ± 0.9‡§ (106)	5.5 ± 1.3§ (60)
Cytochrome b_5 (nmoles/mg)	0.53 ± 0.03	0.58 ± 0.04 (109)	0.30 ± 0.02†§ (57)	0.41 ± 0.03§ (77)	0.52 ± 0.03‡ (98)	0.39 ± 0.02†‡§ (74)
NADPH-cytochrome *c* reductase (mM change in cytochrome *c*/min/mg)	21.7 ± 0.6	44.2 ± 2.5† (204)	10.3 ± 1.4†§ (48)	39.8 ± 2.0†‡§ (183)	37.9 ± 2.6†‡ (175)	12.6 ± 0.6†§ (58)
Cytochrome P-450 (nmoles/mg)	0.52 ± 0.03	1.38 ± 0.07† (265)	0.22 ± 0.02†§ (42)	0.90 ± 0.05†‡§ (173)	0.89 ± 0.06†‡§ (171)	0.25 ± 0.03†§ (48)
Cytochrome P-420 (nmoles/mg)	0	0	0.11 ± 0.07	0	0	0.07 ± 0.03

* All values are mean ± S.E. from three to five separate microsomal preparations. Each microsomal preparation was made from pooled livers of two rats. The number in parentheses is the relative value to normal control rats as 100. Daily drug treatment in phenobarbital groups began 3 days prior to adjuvant administration at a dose of 75 mg/kg and continued for 15 days thereafter. Fenclorac was administered daily for 16 days after adjuvant administration. The control animals received distilled water in volumes similar to that of drug-pretreated groups. All animals were deprived of food 16–18 hr prior to sacrifice, with water *ad lib*.

† $P < 0.05$, compared to normal controls.

‡ $P < 0.05$, compared to adjuvant control group.

§ $P < 0.05$, compared to phenobarbital in normal rats.

reductions in HMDS in adjuvant control and fenclorac-treated rats (Table 2).

The results of this study suggest that the pathogenesis of experimentally induced adjuvant disease in rats involves at least two processes in the polyarthritic response: an immunologic mechanism [18] and a systemic metabolic lesion (hypoactivity of the HMDS). The immunologic mechanism is usually associated with a latent period of 10–16 days from adjuvant administration to the appearance of the inflammatory and osteogenic phases of the disease [18], and is not responsive to pretreatment with an inducer of HMDS (Table 1). This phenomenon is illustrated by the marginal improvements in paw edema and a concomitant complete protection of HMDS from impairment by treatment of diseased animals with phenobarbital. The second process involves the inactivation of hepatic (and possibly extrahepatic) drug-metabolizing enzymes and is responsive to induction by phenobarbital. These results are in agreement with those of Beck and Whitehouse [9] and Zak *et al.* [6], but disagree with those of Morton and Chatfield [5] who did not observe enzyme induction after administration of a single dose of phenobarbital at day 13 post-adjuvant. In rats either with developing or established adjuvant disease, phenobarbital provided protection or marked amelioration for all the components of the hepatic microsomal drug-metabolizing enzymes [10]. These inductive effects of phenobarbital in rats with established disease were evidenced even after a single dose of the drug, with the complete reversal of HMDS to control levels after 3 days of inducer treatment. 3-Methylcholanthrene, another inducer of hepatic enzymes, was also effective in both the established [10] and developing adjuvant disease,* suggesting that the livers of diseased animals with impaired HMDS are capable of inducing enzymes requiring either cytochrome P-450 or P-448. The decrease in HMDS may be responsible for the observed increase in toxicity [11] and longer biologic half-life [19] of non-steroidal anti-inflammatory compounds in adjuvant disease.

Fenclorac, in the doses used in the present study, does not affect the body weight gains in normal [16] or polyarthritic rats (Fig. 1), but the co-administration of fenclorac with phenobarbital ameliorated body weight gain in polyarthritic animals (Fig. 1), suggesting that this phenomenon may be related to improvements in HMDS of the diseased

* P. P. Mathur, personal observation.

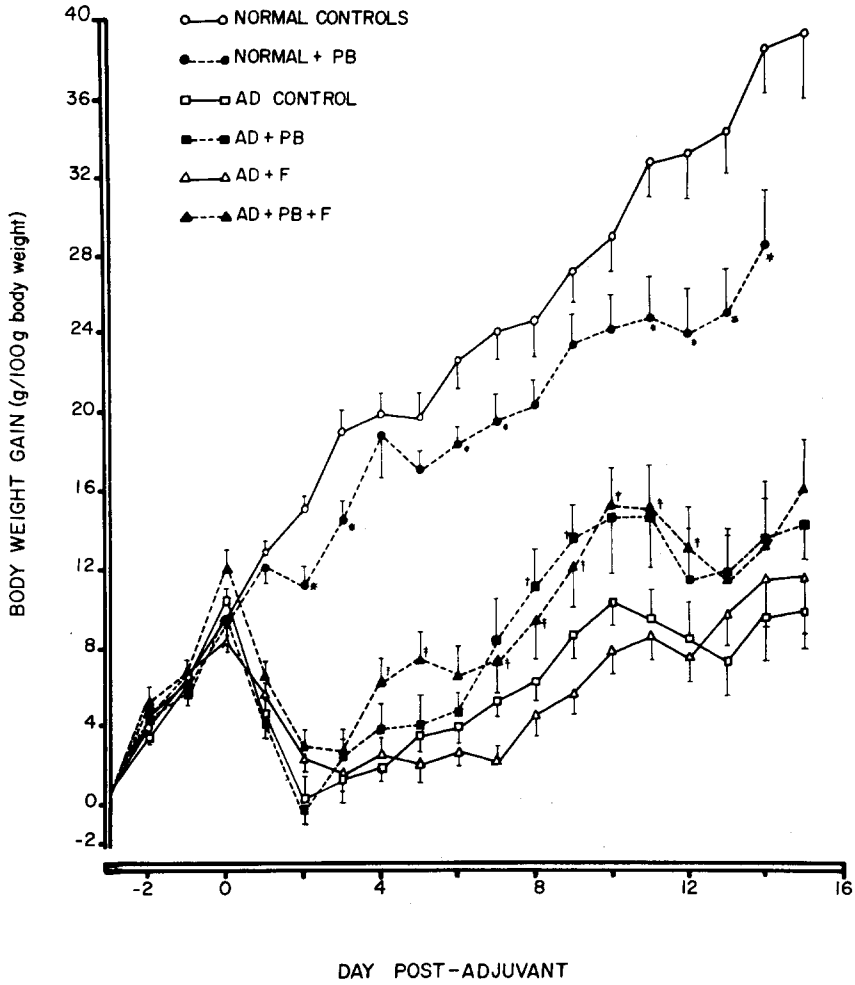

Fig. 1. Effect of phenobarbital and fenclorac on body weight gain in rats with developing adjuvant disease. The adjuvant was administered on day 0. Daily drug treatment began on day −3 in animals receiving phenobarbital (75 mg/kg/day) and on day 0 in animals receiving fenclorac (3 mg/kg/day) and continued for the duration of the experiment. Normal and adjuvant control groups received distilled water as a dosing-vehicle control in volumes similar to that of drug-treated groups. All values are mean ± 1 S.E. from seven to twelve animals. Notice the uniform increase in body weight gain in all groups for the initial 3 days (day −3 to day 0) and an immediate loss in body weight gain in all groups of animals receiving adjuvant. The values in all adjuvant groups were significantly lower when compared to normal controls at all time intervals with $P < 0.05$ or better.

Abbreviations: PB, phenobarbital; AD, adjuvant; and F, fenclorac. The asterisk (*) indicates $P < 0.05$ or better, compared to normal controls; the single dagger (†), $P < 0.05$ or better, compared to AD; and the double dagger (‡), $P < 0.05$ or better, compared to AD+F.

animals. Also, the increases in the activities of drug-metabolizing enzymes in hepatic and possibly in extra-hepatic tissues would be accompanied by increased metabolism and subsequent clearance of both the administered xenobiotics and the substances produced (or the lack of them) within the body in response to various stimuli (unknown) owing to the diseased state of the animals.

Another mechanism of the inhibition of HMDS by adjuvant may involve components of the immune response system. The administration of adjuvant activates a series of immune reactions, resulting in stimulation of the reticuloendothelial system with hepatosplenomegaly and macrophage activation [20, 21]. The macrophage activation during phagocytosis has been shown to stimulate hydrogen peroxide [21] and superoxide [22] production, with subsequent damage of microsomal membrane phospholipids [23, 24] and components of the electron transport chain of HMDS. The temporal pattern of appearance of polyarthritis and the impairment in HMDS would indicate that the changes in HMDS, which were evident as early as 8–14 hr after adjuvant [8, 10] were involved in the primary immunological phase of the disease. Furthermore, it has been demonstrated recently that repeated injections of superoxide dismutase in rats suppressed the carrageenan-induced paw edema [25], and the production of macrophages was inhibited by various nonsteroidal anti-inflammatory agents [26], thus suggesting participation of superoxide radicals in maintaining this phase of inflammation.

Although the HMDS in adjuvant rats receiving phenobarbital was similar to that of a normal rat, the values were lower than those observed in phenobarbital-treated normal animals. This would also suggest that the immunologic events elicited by adjuvant administration may have some role in the reduced activity of HMDS. Fenclorac significantly suppressed the polyarthritic syndrome (an immunologic event), but did not change the impairment in

HMDS; combined therapy with fenclorac and phenobarbital provided protection for both the articular (inflammation) and extra-articular (impairment of HMDS) manifestations of adjuvant disease.

The clinical implications of adjuvant-induced impairment in HMDS and the potential use of combined therapy with an enzyme inducer and nonsteroidal anti-inflammatory agent must be considered in relation to changes in the clinical pharmacological profile of the drug substances. Enzyme induction and drug interaction could significantly alter therapeutic and toxicological activity by changing the rates of metabolism and clearance of the anti-inflammatory drug substance.

Acknowledgements—The authors wish to acknowledge Miss M. A. Reilly for her excellent technical assistance and Mr. George W. Nuss for studies on paw volume.

William H. Rorer, Inc. PERSHOTTAM P. MATHUR*
Research Laboratories Division, ROBERT D. SMYTH†
Fort Washington, PA 19034, CHARLOTTE M. WITMER
U.S.A. GARY S. CARR
and
Department of Pharmacology,
Jefferson Medical College of
Thomas Jefferson University,
Philadelphia, PA 19107, U.S.A.

* Present address: A. H. Robins Co., 1211 Sherwood Ave., Richmond, VA 23220.

† Present address: Bristol Laboratories, P.O. Box 657, Syracuse, NY 13201.

REFERENCES

1. E. M. Glenn and J. Gray, *Am. J. vet. Res*, **26,** 1180 (1965).
2. C. A. Winter and G. W. Nuss, *Arthritis Rheum.* **9,** 394 (1966).
3. B. B. Newbould, *Br. J. Pharmac. Chemother.* **21,** 127 (1963).
4. C. A. Winter, E. A. Risley and G. W. Nuss, *Proc. Soc. exp. Biol. Med.* **111,** 544 (1962).
5. D. W. Morton and D. H. Chatfield, *Biochem. Pharmac.* **19,** 473 (1970).
6. S. B. Zak, F. Honc. and G. Lukas, *Fifth Int. Cong. Pharmac.* (Abstr. No. 1549), 259 (1972).
7. R. P. Carlson and E. I. Ciaccio, *Biochem. Pharmac.* **24,** 1893 (1975).
8. P. P. Mathur, R. D. Smyth, C. Witmer, G. W. Nuss and G. S. Carr, *Fedn Proc.* **35,** 537 (1976).
9. F. J. Beck and M. W. Whitehouse, *Biochem. Pharmac.* **22,** 2453 (1973).
10. P. P. Mathur, R. D. Smyth, C. M. Witmer, G. S. Carr and N. H. Reavey-Cantwell, *J. Pharmac. exp. Ther.*, **203,** 169 (1977).
11. R. D. Sophia, *Pharmacologist* **18,** 219 (1976).
12. G. W. Nuss, R. D. Smyth, C. H. Breder, M. Hitchings, G. N. Mir and N. H. Reavey-Cantwell, *Agents Actions* **6,** 735 (1976).
13. R. D. Smyth, P. P. Mathur, R. L. Procaccini, G. S. Carr and N. H. Reavey-Cantwell, *Toxic. Appl. Pharmac.* **38,** 507 (1976).
14. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
15. P. P. Mathur, R. D. Smyth, T. Herczeg and N. H. Reavey-Cantwell, *J. Pharmac. exp. Ther.* **196,** 204 (1976).
16. P. P. Mathur, R. D. Smyth, C. Witmer, G. Carr and N. H. Reavey-Cantwell, *Curr. Ther. Res.* **21,** 550 (1977).
17. G. W. Snedecor and W. G. Cochran, in *Statistical Methods*, 6th Edn, p. 59. Iowa State University Press, Ames, Iowa (1967).
18. B. H. Waksman, C. M. Pearson and J. T. Sharp, *J. Immun.* **85,** 403 (1960).
19. K. Perrey, H. G. Jonen, G. F. Kahl and E. Jahnchen, *J. Pharmac. exp. Ther.* **197,** 470 (1976).
20. J. G. Howard, *Scott. med. J.* **6,** 60 (1961).
21. M. L. Karnovsky, J. Lazdins and S. R. Simmons, in *Mononuclear Phagocytes* (Ed. R. Van Furth), Vol. 2, p. 423. Blackwell Scientific Publications, Oxford (1975).
22. D. B. Drath and M. L. Karnovsky, *J. exp. Med.* **141,** 257 (1975).
23. D. B. Willas, *Biochem. J.* **113,** 333 (1969).
24. T. Kamataki and H. Kitagawa, *Biochem. Pharmac.* **22,** 3199 (1973).
25. Y. Oyanagui, *Biochem. Pharmac.* **25,** 1465 (1976).
26. Y. Oyanagui, *Biochem. Pharmac.* **25,** 1473 (1976).

Biochemical Pharmacology, Vol. 27, pp. 1774–1777.

0006-2952/78/0701-1774$02.00/0

Effects of the *d*- and *l*-isomers of amphetamine on the levels of 3-methoxy-4-hydroxyphenylglycol sulfate in whole rat brain and rat brain regions

(*Received* 2 *May* 1977; *accepted* 26 *October* 1977)

Considerable evidence has accumulated to suggest that amphetamine increases the availability of the catecholamines (norepinephrine and dopamine) at post-synaptic receptors by releasing catecholamines from pre-synaptic nerve terminals or by blocking their reuptake. Aspects of this extensive literature have been reviewed in several recent volumes [1–3]. However, studies of the effects of acute administration of amphetamine or methamphetamine on the turnover of norepinephrine have yielded variable results. In some studies, norepinephrine turnover in whole brain or brain regions has been found to be increased by amphetamine, as determined by the rate of disappearance of intracerebrally injected [^{3}H]norepinephrine [4–7], while in others norepinephrine turnover in brain appeared to be decreased or unchanged as determined by the accumulation of norepinephrine synthesized *in vivo* from labeled precursors [8–10] or by the rate of depletion of endogenous norepinephrine after synthesis inhibition [11].

The methods used in previous studies to examine the effects of amphetamine on norepinephrine turnover involved various pharmacological or physiological interventions. Therefore, in order to explore this problem further, we have examined the effects of both the *d*- and *l*-isomers of amphetamine on the turnover of norepinephrine in whole rat brain and various regions of rat brain by measuring the endogenous levels of the sulfate conjugate of 3-methoxy-4-hydroxyphenylglycol (MHPG-SO_4), a major metabolite of norepinephrine in rat brain [12, 13]. This