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Studies on the Mechanism of Fat-Free Diet (FFD) Protection Against Indomethacin-Induced Intestinal Ulcers (40130)

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Available evidence suggests that in several animal species, the incidence of indomethacin-induced intestinal ulcers depends upon the extent of enterohepatic circulation (1). The rat in which extensive enterohepatic circulation occurs (1), is particularly sensitive to intestinal lesions (2).

While a chemical or a mechanical interruption of enterohepatic circulation can be held responsible for the protection afforded by cholestyramine or bile duct ligation respectively (3), the mechanism(s) responsible for the protective effect exerted by FFD (4) has not been elucidated. Since Brodie *et al.* (3) correlated incidence of intestinal lesions to rate of bile flow and our data indicated that FFD does not modify bile flow or composition, we thought it worthwhile to determine whether or not such a phenomenon could be attributed to a reduction in intestinal β glucuronidase which could in turn result in a decreased deconjugation of indomethacin.

Preliminary results suggesting that such a reduction occurred, prompted us to determine the possible influence of D-glucaro 1,4 lactone, a known inhibitor of intestinal β glucuronidase (5), on indomethacin-induced intestinal lesions.

Materials and methods. General. Male and female rats, Wistar-Morini strain, weighing 160–180 g were divided into groups of six to ten animals each and had free access to food and water. Thereafter the animals were individually housed in plastic cages with a wire bottom to minimize coprophagy.

Indomethacin (suspended in an aqueous vehicle consisting of NaCl 0.9%, carboxymethylcellulose 0.5%, polysorbate 80 0.5% and benzyl alcohol 0.9%) was administered orally at the dose of 12 mg/kg in a volume of 5 ml/kg. Such a dose was selected which had been shown to induce intestinal ulcers in 90–100% of the animals (4). Controls, where needed, received a similar amount of suspending vehicle.

a) *Influence of nature of the diet on bile flow.* The animals were fed either a regular (RD) or a fat free (FFD) diet over a 10-day period before bile duct cannulation which was performed under ether anesthesia according to a procedure described elsewhere (6). Indomethacin was administered 30 min before bile duct cannulation. After the operation the animals were placed in individual restraining cages. Thirty to forty minutes were usually required for establishment of a constant bile flow, which was thereafter determined over a 6-hr period. The amount of cholic, deoxycholic and chenodeoxycholic acid in bile samples was determined according to Feher *et al.* (7).

b) *Influence of indomethacin and FFD on intestinal β-glucuronidase activity.* The animals were fed either a regular (RD) or a fat free (FFD) diet as described in $\neq$ a. Groups of 6 rats each were killed at different time intervals after indomethacin. Collection of samples and determination of enzyme activity was performed according to Marselos *et al.* (5) with slight modifications. Briefly, an equal amount (0.2 g) of the intestinal content was collected from the jejunum, the ileum and the colon. The pooled samples were homogenized in a Potter–Elvehjem apparatus in 4 vol (w:v) of 0.1% Triton X-100 and centrifuged at 2000*g* for 15 min. Enzyme assays were carried out by incubating for 1 hr at 37° 200 μl of the supernatant with 300 μl of 1.5 m*M* phenolphtalein β glucuronide dissolved in 66.7 m*M* phosphate buffer at pH 7. The blank was prepared in a similar manner without incubation. The reaction was stopped by adding 0.1 ml of 25% trichloroacetic acid. The optical density of the released phenolphthalein was measured at 540 nm. The protein content was determined according to a modification of the Lowry method (8). The enzyme activity was expressed as nmoles of phenolphthalein formed per min and per mg of protein.

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c) *Influence of D-glucaro 1,4 lactone on intestinal β glucuronidase activity.* The animals were fed a regular (RD) diet as described in ≠ a. D glucaro 1,4 lactone was administered orally in two doses of 600 mg/kg each at 3 and 6 hr after indomethacin. Groups of 6 rats each were killed 8 hr after indomethacin and intestinal β glucuronidase activity determined as described in ≠ b.

d) *Influence of D glucaro 1,4 lactone on degree of intestinal ulcers.* RD rats were given orally D glucaro 1,4 lactone in two doses of 600 mg/kg each at 3 and 6 hr after indomethacin. Groups of 10 animals each were sacrificed by CO_2 asphyxiation 72 hr after indomethacin and the intestine carefully examined for presence of ulcers by an observer unaware of the treatment.

e) *Mechanism(s) by which FFD and D-glucaro 1,4 lactone inhibit intestinal β glucuronidase activity.* Groups of 4 RD or FFD and D glucaro 1,4 lactone treated RD rats were killed 8 hr after indomethacin and intestinal β glucuronidase activity determined as described in ≠ b. Vm and Km values were calculated according to Eisenthal *et al.* (9) by plotting different substrate concentration (0.2, 0.4, 0.8, 1.6 mmoles of phenolphtalein β glucuronide) against the values of the relative velocities (expressed as nmoles phenolphthalein formed/min/mg protein). Type of inhibitory mechanism(s) was determined according to Gillette (10).

f) *Statistics.* The data were analyzed according to the following methods: (i) Student's *t* test (11): those relative to bile flow and composition as well as effect of D glucaro 1,4 lactone on intestinal ulcers and β glucuronidase activity. (ii) Analysis of variance (12): those relative to the effect of indomethacin and FFD on β glucuronidase activity. When analysis of variance indicated significant *F* value, inspection of all differences between pairs of means was made according to the LSD method. (iii) Nonparametric 95% confidence limits for *Km* and *Vm* median values were calculated according to Colquhoun (13).

Results. Statistical analysis of data in Table I indicates that: a) rate of bile flow is independent of nature of the diet and b) the effect of FFD is confined to a moderate but significant increase in chenodeoxycholic acid concentration. Statistical evaluation of data in Fig. 1 indicates that: (i) presence of indomethacin is conditio sine qua non for modifications in intestinal β glucuronidase activity to occur in either RD or FFD animals. (ii) enzyme activity is moderately but significantly increased only 8 hr after indomethacin in RD rats while it is markedly reduced at

TABLE I. INFLUENCE OF NATURE OF THE DIET ON BILE FLOW OF THE UNANESTHETIZED RAT. BILE WAS COLLECTED OVER A 6 HR PERIOD.

Type of diet	No of animals	Average bile flow (μl/min) mean ± SE
Regular (RD)	24	12.1 ± 0.33
Fat free (FFD)	24	11.5 ± 0.31

INFLUENCE OF NATURE OF THE DIET ON BILE ACIDS CONCENTRATION (μg/100 ml) OF UNANESTHETIZED RAT.

Type of diet	No of animals	Deoxycholic acid mean ± SE	Cholic acid mean ± SE	Chenodeoxycholic acid mean ± SE
Regular (RD)	8	4.920 ±0.239	15.289 ±0.695	7.415 ±0.197
Fat free (FFD)	8	5.447 ±0.448	14.134 ±0.513	8.394[a] ±0.262

[a] $P < 0.05$ as compared to RD rats.

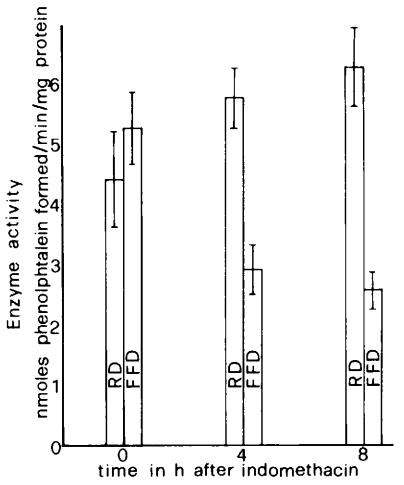

FIG. 1. Influence of a fat free diet on intestinal β-glucuronidase after oral indomethacin (12 mg/Kg). Mean ± SE (6 animals per group). Results of the LSD method: S^2 = mean square within treatments = 2.13 $LSD_{0.05}$ = difference between two means required for 5% significance = 1.81 $LSD_{0.01}$ = difference between two means required for 1% significance = 2.40.

TABLE II. EFFECT OF D-GLUCARO 1,4 LACTONE (1.2 g/kg) ON DEGREE OF INTESTINAL ULCERS AND β-GLUCURONIDASE ACTIVITY OF INTESTINAL CONTENT AFTER ORAL INDOMETHACIN (12 mg/kg) IN THE RAT.

Treatment	Ulceration degree Index[a]	% inhibition	Enzyme activity[b]	% inhibition
Indomethacin	2.8 (10)	—	7.8 ± 0.5[e] (8)	—
Indomethacin + D-glucaro 1,4 lactone	1.5[c] (10)	46	4.6[d] ± 0.2[e] (6)	41

[a] Degree of intestinal ulceration was graded according to an arbitrary scale: 0 = normal; 1 = primary ulcer; 2 = advanced ulcer; 3 = perforating ulcer and intestinal adhesions; 4 = death from intestinal lesions.
[b] Expressed as nmoles phenolphthalein formed/min/mg protein.
[c] $P < 0.05$ as compared to indomethacin.
[d] $P < 0.01$ as compared to indomethacin.
[e] Mean ± SE in parentheses number of animals.

TABLE III. MECHANISM OF INHIBITION OF FFD AND D GLUCARO 1,4 LACTONE ON INTESTINAL β GLUCURONIDASE ACTIVITY.

Inhibitor	*Vm* median value[a] and its 95% confidence limits	*Km* median value[b] and its 95% confidence limits	Mechanism of inhibition
—	8.25 (9.25–6.50)	0.11 (0.20–0.02)	—
FFD	3.90 (5.55–3.10)	0.64 (1.35–0.41)	Mixed
D glucaro 1,4 lactone	6.20 (7.00–6.00)	0.34 (0.41–0.30)	Competitive

[a] Expressed as nmoles phenolphthalein formed/min per mg protein.
[b] Expressed as mmoles of phenolphthalein β glucuronide. See materials and methods for details.

both 4 and 8 hr after the administration of the antiphlogistic agent in FFD animals.

Data in Table II indicate that D-glucaro 1,4 lactone significantly reduces both degree of intestinal lesions and β glucuronidase activity. The determination of the mechanism(s) by which FFD and D-glucaro 1,4 lactone inhibit intestinal β glucuronidase activity, indicates (Table III) the following antagonisms: (i) competitive for D-glucaro 1,4 lactone, insofar as the apparent *Km* is significantly increased and the apparent *Vm* is unchanged (ii) mixed for FFD, insofar as the apparent *Km* is significantly increased and the apparent *Vm* is decreased.

Discussion. Intestinal persistence of free indomethacin, partly as a result of enzymic deconjugation of recycled indomethacin glucuronide (14, 15), has been held responsible for the development of intestinal lesions (1, 16) probably through a disruption of the mucosal barrier (17).

Of the many factors involved, Brodie *et al.* (3), emphasized the importance of bile and correlated the incidence of intestinal lesions to the rate of bile flow. However, as in the case of regular diet (RD) fasted animals (18), our data indicate that the lower incidence of indomethacin-induced intestinal lesions observed in fat free diet (FFD) rats (4), must be attributed to factor(s) other than a decrease in bile flow or bile acid concentration. Experimental evidence rather suggests that the significant drop in β glucuronidase activity observed in indomethacin-treated FFD rats might play a role in reducing intestinal ulcers. This is substantiated by the observation that D-glucaro 1,4 lactone reduces β glucuronidase activity as well as incidence of intestinal lesions. It should be mentioned here that, in agreement with the data of Fischer *et al.* (19), β glucuronidase as determined in our experiments is of bacterial rather than mammalian origin, since no activity (unpublished results) was found at pH 5 which is the optimal condition for the mammalian enzyme (5, 20).

In view of Kent's observation that indomethacin causes intestinal flora overgrowth (21) and our data on FFD indicating the existence of an inhibitory mechanism of mixed type, it appears legitimate to assume that indomethacin-induced increase or decrease in β glucuronidase activity in RD and FFD rats respectively, might reflect alterations in the intestinal microflora. These changes involve mainly those microorganisms such as Bacteroides and Clostridium (21) known to produce β glucuronidase (22). Correlation between microbial β glucuronidase and intestinal lesions is further substantiated by the observations of Kent *et al.* indicating that a combination of neomycin, polymixin B and bacitracin markedly reduces indomethacin-induced intestinal lesions (21)

as well as intestinal β glucuronidase activity (22). Without any doubt a decreased deconjugation of indomethacin glucuronide or a reduction in intestinal microflora which could act synergistically with indomethacin in causing the initial mucosal damage (21) or both, must play a role in reducing intestinal ulcers in FFD rats.

The mechanism(s) by which FFD reduces the incidence of indomethacin-induced intestinal lesions cannot be fully explained on the basis of our present data.

The possibility that other factors might play a significant concomitant role must not be overlooked.

Summary. The possible relationship between bile flow and/or composition as well as β glucuronidase activity of the intestinal content and indomethacin-induced intestinal lesions has been investigated.

In general, the following conclusions can be drawn: (i) The lower incidence of intestinal lesions in fat free (FFD) as compared to regular (RD) diet rats, must be attributed to factor(s) other than reduction in bile flow and/or composition. (ii) Intestinal β glucuronidase activity is markedly reduced in indomethacin-treated FFD and D-glucaro 1,4 lactone medicated RD rats. (iii) In agreement with data in the literature, our results indicate that there is a relationship between the decrease in intestinal β glucuronidase activity and the reduction in intestinal lesions. These findings are discussed.

1. Duggan, D. E., Hooke, K. F., Noll, R. M., and Kwan, K. C., Biochem. Pharmacol. **26,** 1749 (1975).
2. Wilhelmi, G., Pharmacology **11,** 220 (1974).
3. Brodie, D. A., Cook, P. G., Bauer, B. J., and Dagle, G. E., Toxicol. Appl. Pharmacol. **17,** 615 (1970).
4. Volterra, G., Pisanti, N., and Meli, A., Proc. Soc. Exp. Biol. Med. **146,** 146 (1974).
5. Marselos, M., Dutton, G., and Häänninen, O., Biochem. Pharmacol. **24,** 1855 (1975).
6. Meli, A., and Riva, M., Il Farmaco Ediz. Pratica **26,** 269 (1971).
7. Feher, T., Papp, J., and Kazik, M. H., Z. Klin. Chem. Klin. Biochem. **11,** 376 (1973).
8. Wang, C. S., and Smith, R. L., Anal. Biochem. **63,** 414 (1975).
9. Eisenthal, R., and Cornish Bowden, A., Biochem. J. **139,** 715 (1974).
10. Gillette, J. R., in "Fundamentals of drug metabolism and drug disposition (B. M. La Du, H. G. Mandel, and E. L. Way, eds) p. 407, Williams and Wilkins, Baltimore 1971.
11. Snedecor, G. W., and Cochran, W. G., "Statistical Methods" p. 103 The Iowa State University Press 1972.
12. Ibid p. 258; p. 272.
13. Colquhoun, D., "Lectures on Biostatistics" p. 103 Clarendon Press Oxford 1971.
14. Yesair, D. W., Callahan, M., Remington, I., and Kensler, C. J., Biochem. Pharmac. **19,** 1579 (1970).
15. Hucker, H. B., Zacchei, A. G., Cox, S. V., Brodie, D. A., and Catwell, N. H. R., J. Pharmac. Exp. Therap. **153,** 237 (1966).
16. Duggan, D. E., Hogans, A. F., Kwan, K. C., and McMahon, F. G., J. Pharmac. Exp. Therap. **181,** 563 (1972).
17. Chvasta, T. E., and Cooke, A. R., J. Lab. Clin. Med. **79,** 302 (1972).
18. Del Soldato, P., and Meli, A., Toxicology in press. (1978).
19. Fischer, L. J., Kent, T. H., and Weissinger, J. L., J. Pharmacol. Exp. Ther. **185,** 163 (1973).
20. Scheline, R. R., Pharmacol. Reviews **25,** 451 (1973).
21. Kent, T. H., Cardelli, R. M., and Stamler, F. W., Amer. J. Pathol. **54,** 237 (1969).
22. Kent, T. H., Fischer, L. J., and Marr, R., Proc. Soc. Exp. Biol. Med. **140,** 590 (1972).

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