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INHIBITION OF THE RELEASE OF PROSTAGLANDINS, LEUKOTRIENES AND LYSOSOMAL ACID HYDROLASES FROM MACROPHAGES BY SELECTIVE INHIBITORS OF LECITHIN BIOSYNTHESIS

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Abstract—The release of the inflammatory mediators, prostaglandins (PGs), leukotrienes (LT) and lysosomal acid hydrolases (LAH), by macrophages is stimulated by endocytic stimuli such as zymosan. This process can be interfered with by specific inhibitors of phosphatidylcholine (PC) biosynthesis. The diphenylsulfone dapsone and three analogs selectively inhibited [^{14}C]choline incorporation into PC but had varied effects on inhibition of mediator release by macrophages. Dapsone inhibited the release of PGs, LT and LAH, whereas the three closely related structural analogs inhibited LT and LAH release only, with little or no effect on PG production.

Mononuclear phagocytes maintained in culture release a variety of products into the medium. These products include prostaglandins [1–3], leukotrienes C_4 and B_4 [4, 5], lysosomal acid hydrolases [6, 7], lysosomal phospholipase A_2 [8], neutral proteinases [9–11], factors which stimulate lymphocytes [12, 13], and various complement components [14]. Three of these products, prostaglandins, leukotrienes and lysosomal acid hydrolases, are released from cultured mouse peritoneal macrophages when exposed to zymosan [1–5]. The release of prostaglandins, but not of leukotrienes or lysosomal acid hydrolases, is inhibited by the addition of the non-steroid anti-inflammatory compound indomethacin (Ref. 2 and J. L. Humes, unpublished). Since the release of products from cultured cells undergoing phagocytosis certainly involves events mediated by the plasma membrane, it was of interest to study the effects of inhibitors of phospholipid synthesis on these secretory processes. In a recent report we have shown that two diphenylsulfones, dapsone and AUS*, specifically inhibit the biosynthesis of phosphatidylcholine (PC) from precursor choline and that this is the major route of synthesis of PC in mouse macrophages [15]. The activities of these compounds are reversible and are not mediated through toxicity [15]. We now show that dapsone and AUS, as well as two analogs of AUS, inhibit the release of lysosomal acid hydrolases and the synthesis of leukotriene induced by the phagocytosis of zymosan. On the other hand, only dapsone inhibits the release of all three classes of mediators, PG, acid hydrolases and leukotrienes.

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* Abbreviations: AUS, 1-[4-(4-sulfanilyl)phenyl]urea; LAH, lysosomal acid hydrolases; PC, phosphatidylcholine; AA, arachidonic acid; DMEM, Dulbecco's modified Eagle's medium; HIPS, heat-inactivated porcine serum; NAG, *N*-acetyl-β-D-glucosaminidase; LTD_4, leukotriene C_4; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; $6KF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; TCA, trichloroacetic acid; and HPLC, high pressure liquid cromatography.

MATERIALS AND METHODS

Materials. Male Swiss–Webster mice (HLA-SW/ICR SPF) were purchased from Hilltop Lab Animals, Inc. Scottsdale PA. M199 medium, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and porcine serum were bought from the Grand Island Biological Co., Grand Island, NY. The porcine serum was inactivated by heating at 56° for 30 min (HIPS). Nunclon tissue culture dishes were from Vanguard International, Inc., Neptune, NJ. [5,6,8,9,11,12,14,15-^{3}H (N)]Arachidonic acid (60 Ci/mmole) and [1,2-^{14}C]choline (64 mCi/mmole) were from the New England Nuclear Corp., Boston, MA. Zymosan was from ICN K & K Laboratories, Inc., Plainfield, NY. The zymosan particles were suspended in phosphate-buffered saline (PBS), boiled, and centrifuged three times. The final pellet was suspended in PBS at a concentration of 20 mg/ml and stored frozen. The zymosan suspensions were thawed, diluted, and sonicated before addition to the cultures. Thioglycollate broth was supplied by BBL, Cockeysville, MD, and dissolved as described on the label. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide was from the Sigma Chemical Co., St. Louis, MO, and phenolphthaleinglucuronide from CalBiochem, San Diego, CA. PGE_2 was purchased from ONO Pharmaceuticals, Osaka, Japan; SG-81 chromatographic paper was from Ace Scientific, Linden, NJ.

The structures of the four compounds are shown

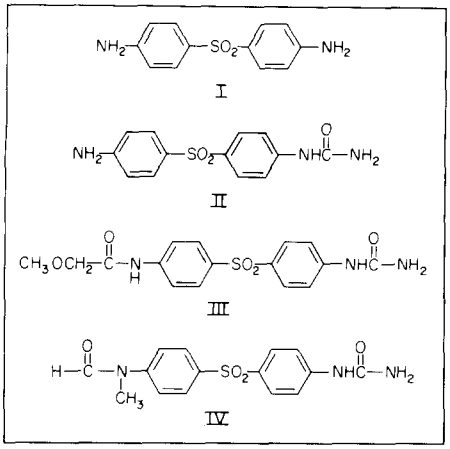

Fig. 1. Structures of dapsone (I), AUS (II) and analogs (III and IV).

in Fig. 1. Dapsone (I) was obtained from the Aldrich Chemical Co. (mol. wt 248.31). 4-Methoxyacetamido-4′-ureidodiphenyl sulfone (III) was prepared from methoxyacetyl chloride and II (mol. wt 291.33) in pyridine by known procedures [16] and recrystallized from methanol to give III (mol. wt 363.4) (m.p. 222–224°). 4-(*N*-Methylformamido)-4′-ureidodiphenyl sulfone (IV) was prepared by reducing 4-formamido-4′-nitrodiphenyl sulfone [17] with diborane in tetrahydrofuran to give 4-methylamino-4′-nitro-diphenyl sulfone (m.p. 227–230°). Treatment of this material in refluxing 97–100% formic acid gave 4-(*N*-methylformamido)-4′-ureidodiphenyl sulfone (m.p. 134–137°), which was reduced catalytically in ethyl acetate solution, at 3 atmospheres of hydrogen, in the presence of Raney-Nickel to give 4-(*N*-methylformamido)-4′-aminodiphenyl sulfone (m.p. 158–160°). Conversion of this material to the ureido product IV was accomplished by successive treatment with phosgene and ammonia using known procedures [18]. Recrystallization from ethanol gave IV as an ethanol solvate (m.p.: foams at 134–137°, melts at 191–193°; mol. wt 379.44).

Synthetic LTC_4 was obtained from Dr. J. Rokach of Merck Frosst Canada Laboratories and stored as a frozen aqueous solution [19].

Tissue culture. Macrophages were collected by peritoneal lavage from untreated mice and from mice which 4 days previously had received an intraperitoneal injection of 2 ml of thioglycollate broth. The lavage medium was M199 containing 100 units/ml of penicillin and streptomycin, 20 units/ml heparin, and 1% HIPS. The cells were plated at 4×10^6 per 60 mm culture dish and incubated for 2 hr at 37° in 5% CO_2 in air. The nonadherent cells were removed by washing the cell sheet four times with PBS, and the adherent cells were maintained overnight in M199 + 10% HIPS.

Harvest of media and cells. At the termination of the experiment, the media were collected and divided for subsequent analysis. Isotonic saline (2 ml) containing 0.1% Triton X-100 was added to each plate, and the lysed cells were removed with a rubber policeman.

Enzyme assays. Lactate dehydrogenase was assayed by determining the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm. *N*-Acetyl-β-D-glycosaminidase (NAG) was assayed by the method of Woolen *et al.* [20] using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate dissolved in 0.1 M citrate–phosphate buffer (pH 4.5) and β-glucuronidase as assayed by the method of Talalay *et al.* [21]. A unit of enzyme activity for NAG and β-glucuronidase is defined as the amount of enzyme needed to cleave 1 nmole of substrate per hr at 37°. Elastase was assayed as previously described [11].

Assay for prostaglandins and leukotrienes. Macrophages were incubated with 1.5 μCi of [^{3}H]AA in 1 ml M199 containing 1% HIPS. After 4 or 20 hr, the radioactivity labeled cells were washed twice with 2 ml of M199 containing 1% HIPS, and various additions were made in 1 ml of this tissue culture medium [5]. After the incubation period (usually 3 hr), the media were collected, buffered at pH 4.7 with 0.1 M sodium acetate, and extracted with two 3-ml aliquots of freshly distilled diethyl ether. The aqueous phases were then reextracted with 3.75 ml chloroform/methanol (1/2), 1.25 ml chloroform and 1.25 ml water. The ether phases were evaporated to dryness under a stream of nitrogen at 40°. The residues were dissolved in 0.2 ml of ethyl acetate/methanol (3/1), and aliquots (usually 0.1 ml) were applied to 1 cm × 20 cm lanes of a thin-layer chromatography plate. Aliquots of the aqueous phase (usually 0.1 ml) were similarly spotted. The plates were developed with ethyl acetate/methanol/acetic acid (95/5/1), dried, and then redeveloped in the same direction with hexane/ethyl ether/acetic acid (60/40/1). The origin (the cellulose spotting gel) containing the LTC_4 from the chromatography of the aqueous phase and the PGE_2 zones from the chromatograph of the ethyl ether extracts were removed, and the radioactivity was determined in 8 ml of Aquasol with a Packard 3255 liquid scintillation spectrometer. The percentage recoveries of [^{3}H]PGE_2 and [^{3}H]LTC_4 (prepared by HPLC from extracts of macrophage culture media) were 80 ± 7 and 69 ± 2 (N = 3) respectively [5].

RESULTS

Inhibition of choline incorporation by dapsone and analogs. We have shown previously that the biosynthetic pathway for phosphatidylcholine synthesis from choline could be selectively inhibited by dapsone and AUS [15]. We have now extended this series of diphenylsulfones to include two new analogs, III and IV, which are also capable of inhibiting choline incorporation by macrophages. The methoxyacetamido analog (III) showed a dose-dependent inhibition of [^{3}H]choline incorporation into TCA-insoluble material of cultured macrophages (Fig. 2). When the two analogs, dapsone and AUS, were compared at 40 μg/ml, a rank order was established III > AUS > IV and dapsone (Fig. 2). Interference with this pathway could alter membrane phospholipid composition and, subsequently, function. Therefore, we examined the response of macrophages to the model inflammatory stimulus, zymo-

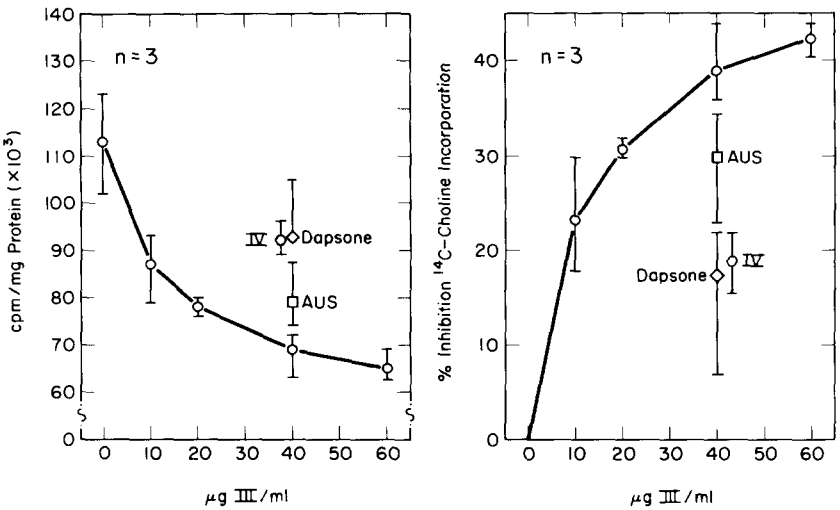

Fig. 2. Effects of dapsone and three analogs on [^{14}C]choline incorporation by macrophages. Macrophages (5×10^6) from resident mice were cultured as described in Materials and Methods. M199 medium (4 ml) containing 1% HIPS and 0.5 μCi of [^{14}C]choline was added to the plates. Dapsone and analogs were dissolved in dimethyl sulfoxide (DMSO) and added as described. All cultures contained a final concentration of 0.1% DMSO. Cells were harvested after 4 hr, and radioactive uptake was determined. The results for AUS, dapsone and IV were taken from separate dose–response curves, and the data at 40 μg/ml were plotted. The results are the averages ± S.D.; N = 3.

san, in the presence and absence of these various sulfones.

Selective release of lysosomal acid hydrolases. The addition of a phagocyte stimulator such as zymosan to cultures of unstimulated mouse macrophages induces the release of lysosomal acid hydrolases [2, 4]. Untreated cells cultured for 4 hr in serum-free medium retain 90–95% of the original total NAG activity, whereas the addition of zymosan causes the release of 50% of the total activity without loss of the cytoplasmic marker enzyme, LDH. Thus, the release of lysosomal enzymes is selective. Zymosan had no effect on the incorporation of [^{3}H]choline into phospholipids. We compared the effect of AUS on choline incorporation and on zymosan-induced release of two lysosomal acid hydrolases of cells isolated from the same group of mice (Table 1). AUS inhibited choline incorporation in control as well as zymosan-treated cultures and significantly decreased the zymosan-induced release of lysosomal acid hydrolases (Table 1). The addition of increasing levels of dapsone (Fig. 3, left panel) or AUS (Fig. 3, right panel) prevented the zymosan-induced release of the enzyme, in a concentration-dependent manner. Compound III was slightly more potent than AUS in inhibiting enzyme release (50 μg/ml of III gave 34% inhibition and 50 μg/ml AUS gave 27% inhibition). Both AUS and III were more potent than dapsone and IV which were about equally effective.

Effects on prostaglandin and leukotriene production. In the same cultures utilized for lysosomal

Table 1. Effect of AUS on the selective release of acid hydrolases from mouse macrophages induced by the inflammatory stimulus zymosan*

	Choline incorporation (cpm/mg protein)	LDH activity (mUnits/ml)		Acid hydrolase release† (% activity in medium)	
		Medium	Cells	β-Glucuronidase	*N*-Acetyl-β-D-glucosaminidase
Control	92,000	0	134 ± 7	6.70 ± 2.58	11.68 ± 1.44
Control + AUS	68,500	0	123 + 7	3.41 + 2.40	11.78 + 1.42
Zymosan	94,000	0	128 + 7	51.51 + 6.27	51.59 + 3.79
Zymosan + AUS	67.900	0	119 + 10	33.42 + 6.91‡	36.63 + 3.53‡

* Macrophages were isolated and cultured as described in Materials and Methods. A group of plates was used to study the effect of AUS on [^{14}C]choline incorporation. The results are the averages of duplicate determinations. Another group of plates was used to measure the effect of AUS on the selective release of lysosomal hydrolases caused by zymosan. AUS was added at 40 μg/ml, and the enzymes were assayed in the media and in cell lysates after 4 hr of incubation. Results are averages ± S.D.; N = 3.

† One hundred percent activity of β-glucuronidase was 51.3 ± 3.6, and of *N*-acetyl-β-D-glucosaminidase was 1381 ± 166, nmoles of substrate per hr.

‡ $P < 0.01$.

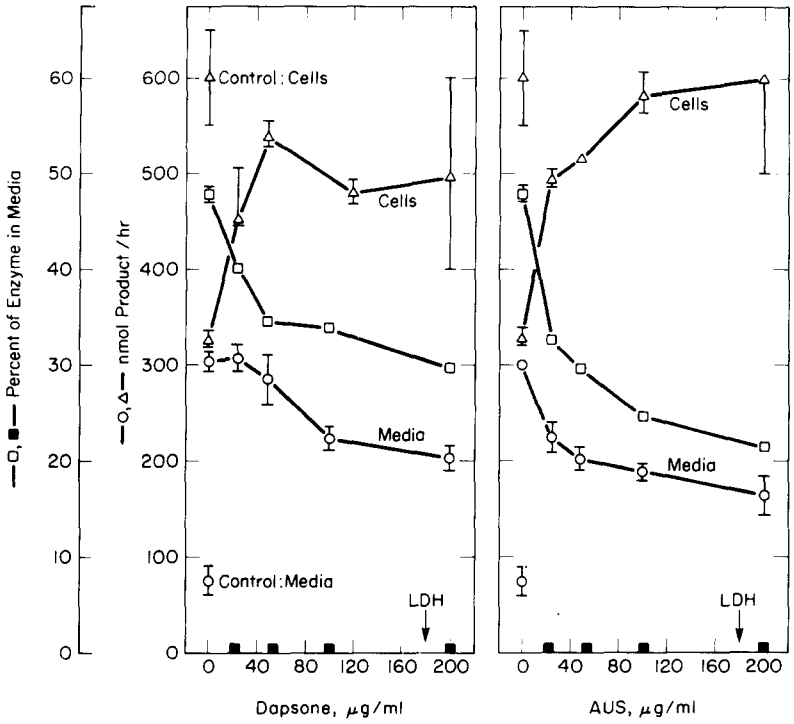

Fig. 3. Inhibition of lysosomal hydrolase release by dapsone and AUS. Resident macrophages were cultured as described in Materials and Methods. The compounds were dissolved in DMSO and added to the cultures for 1 hr. Zymosan (50 μg/ml) was then added to the medium, and the cells were incubated for an additional 4 hr. The activities of *N*-acetyl-β-D-glucosaminidase in the cells (△) and in the media (○), as well as the percentages of the activities in the media (□), were calculated. The percentages of LDH released into the media are also presented (■). Results are averages ± S.D.; N = 3.

Table 2. Selective inhibition by AUS of LTC_4 synthesis*

Expt.	Addition	Concn (μg/ml)	PGE_2 (% Inhibition)	LTC_4 (% Inhibition)
1	AUS	50	+6	37
		100	+24	43
		200	12	45
	Dapsone	50	25	15
		100	51	59
		200	75	64
2	AUS	50	+30	5
		100	+11	11
		200	+7	39
	Dapsone	50	29	35
		100	39	32
		200	64	54
3	AUS	100	6	48
		200	21	61

* Macrophages from untreated mice were cultured as described in Materials and Methods. AUS and dapsone, dissolved in DMSO, were added to the cultures 1 hr before the addition of 50 μg/ml of zymosan. The inhibition of zymosan-induced PGE_2 and LTC_4 by AUS and dapsone was calculated. Each result is the average of duplicate determinations.

One hundred percent zymosan-induced PGE_2 was: Expt. 1—18,253 ± 1,946 cpm; 2—17,078 ± 1,068; 3—16,759 ± 532; and LTC_4 was: 1—359 ± 11 cpm; 2—378 ± 45; 3—220 ± 14.

acid hydrolase measurements, we were able to determine the amount of prostaglandin released into the medium. Relatively little PGE_2 or $6KF_{1\alpha}$ was released into the medium of control cultures during the 4-hr incubation. However, the addition of 50 μg/ml of zymosan induced a large increase in the amount of these prostaglandins released into the medium (Fig. 4). The addition of dapsone to the zymosan-containing medium inhibited, in a concentration-dependent manner, the increased production and release of these compounds (Fig. 4). Unlike the inhibition of acid hydrolase release, AUS was less effective than dapsone in inhibiting PG production, with significant inhibition observed only at the highest dose tested.

A further selectivity of the actions of AUS and dapsone was found when we examined their effects on two products of the arachidonic acid cascade, prostaglandins and leukotrienes. In three experiments AUS inhibited only LTC_4 synthesis and release, whereas in two experiments dapsone inhibited PGE_2 and LTC_4 synthesis and release (Table 2).

Effect of diphenylsulfones on the release of an elastase from elicited cells. Elastase, a neutral proteinase, is released into the medium of elicited, and to a lesser extent resident, mouse macrophages [9–11]. We tested the effect of dapsone on the release

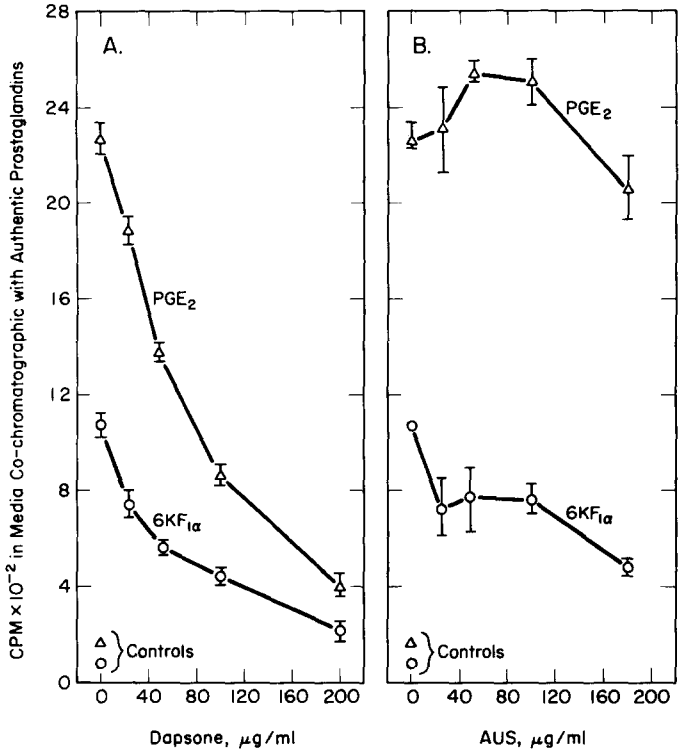

Fig. 4. Effects of dapsone and AUS on zymosan-induced PG release. Resident macrophages, cultured as described in Materials and Methods, were labeled with 1 μCi of [^{3}H]arachidonate for 4 hr and washed free of isotope. Compounds dissolved in DMSO were added to the cultures for 1 hr. Zymosan (50 μg/ml) was added to the cultures, and the cells were incubated for an additional 4 hr. Media and cells were collected for subsequent prostaglandin measurements. The amounts of radioactivity found in the media that co-chromatographed with prostaglandin E_2 and $6KF_{1\alpha}$ was determined. Results are the averages ± S.D. for three determinations.

of elastase from thioglycollate-elicited macrophages. In these experiments, the cells were incubated in DMEM without serum but with 0.2% lactalbumin hydrolysate. As shown in Table 3, dapsone did not affect the release of elastase into the medium. In addition, the compound was non-toxic as measured by the retention of lysosomal acid hydrolases (NAG) and cytoplasmic LDH. Only at the higher concentration of 80 μg/ml for 3 days was there a small, but not statistically significant, increase in LDH activity found in the medium.

DISCUSSION

Zymosan, a preparation of yeast cell walls, has been shown to induce chronic inflammation in mice [7]. This activity is not simply due to its being endocytosed by phagocytic cells, since latex beads

Table 3. Effect of dapsone on elastase release from elicited macrophages*

Additions	Elastase activity [μg [^{3}H]elastin hydrolyzed · ml^{-1} · (18 hr)$^{-1}$]	LDH (mUnits/ml)	
		Cell	Medium
None	88.0 ± 12.5	357 ± 27	28 ± 9
Dapsone, 10 μg/ml	95.5 ± 9.5	365 ± 33	46 ± 21
Dapsone, 20 μg/ml	90.5 ± 5.5	342 ± 15	55 ± 8
Dapsone, 40 μg/ml	85.5 ± 4.0	330 ± 47	58 ± 36
Dapsone, 80 μg/ml	99.5 ± 9.0	303 ± 8	99 ± 4

* Macrophages were isolated from thioglycollate-stimulated mice, and the adherent cells were maintained in Dulbecco's medium (DMEM) ± heat-inactivated acid-treated fetal calf serum for 48 hr. The medium was then changed to DMEM + lactalbumin hydrolysate, and the cells were incubated for 24 hr. The media were collected and assayed for elastase [11] and the cells and media were assayed for LDH content.

Results are averages ± S.D.; N = 3.

of similar size which are readily phagocytosed do not induce inflammation [7]. We have shown previously that cultures of unstimulated macrophages respond to zymosan but not to latex by releasing prostaglandins [1] and lysosomal acid hydrolases [2]. Thus, this system, which is composed almost entirely of macrophages, can be used to study the interactions of inflammatory stimuli with cells under the defined conditions of cell culture.

The synthesis and release of prostaglandins and leukotrienes and the selective release of lysosomal acid hydrolases by macrophages exposed to zymosan certainly involve biochemical processes mediated by the plasma membrane. One way to study this process is to utilize specific reagents which modify the composition of the membrane in a non-toxic manner. We have shown that the diphenylsulfones, dapsone and AUS, specifically inhibit the biosynthesis of lecithin from precursor choline [15]. These chemicals as well as two structural analogs, as shown in this report, inhibit the zymosan-induced release of certain mediators of inflammation. It is of interest that, although AUS was more potent than dapsone in inhibiting choline incorporation ([15] and Fig. 1) and lysosomal acid hydrolase release (Fig. 3), it was less active than dapsone in preventing PG release. (Fig. 4). The reason for this difference between the effects of compounds with such similar structures is unknown. However, it has been shown that release of PGs and that of lysosomal hydrolases from macrophages are independently regulated [22]. Lastly, the differential effects of dapsone and AUS on regulation of the arachidonic acid cascade induced in macrophages by zymosan is also of interest. Dapsone appears to be unique in inhibiting the synthesis and release of both PGs and LTs, whereas AUS blocks LT production but not PG production. It has been proposed by Humes *et al.* [5] that the leukotriene pathway and the cyclooxygenase pathway are independently regulated. The data reported in this paper support that hypothesis.

The parent compound of this series, dapsone, is an established antimalarial and antileprotic agent [23]. This sulfone is also used to treat dermatitis herpetiformis [24], a disease which is characterized by polymorphonuclear infiltration. Although the mechanism of action of dapsone on these diseases is unknown, our findings may shed some light on this subject. Macrophages are capable of releasing the potent chemotactic agent leukotriene B_4 (LTB_4) in response to inflammatory stimuli [5], and leukotriene synthesis can be inhibited by dapsone. Thus, it is possible that the generation of LTB_4 at sites of tissue injury is decreased, thereby reducing the recruitment of inflammatory cells.

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