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THE EFFECTS OF SULPHASALAZINE AND ITS METABOLITES ON PROSTAGLANDIN PRODUCTION BY HUMAN MONONUCLEAR CELLS

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Abstract—Although it has been proposed that sulphasalazine (SASP) and its metabolite 5-aminosalicylic acid (5-ASA) act therapeutically by inhibiting production of vasoactive and immunoregulatory prostaglandins (PGs), in previous *in vitro* studies these drugs have both inhibited and promoted PG production. This study demonstrates that SASP and 5-ASA promote or inhibit peripheral blood mononuclear cell PG production depending upon the PG measured, the concentration of the drug, and whether the cells were stimulated. Sulphapyridine, the other constituent of SASP, only inhibited production. At high concentrations of SASP and 5-ASA the viability of mononuclear cells was reduced. The enhancement of PG production and toxicity was greater with SASP than 5-ASA, while the PGs most affected by SASP were not those most affected by 5-ASA. Thus, *in vitro* SASP may possess properties other than those of 5-ASA and this may explain the different therapeutic properties of these two compounds.

Although sulphasalazine (SASP†) has been used in the treatment of inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) for nearly 50 years, its mechanism of action remains unknown. Whereas in IBD it may act as a pro-drug and deliver the therapeutic moiety, 5-aminosalicylic acid (5-ASA), to the inflamed colon, in RA 5-ASA is ineffective and the therapeutic properties may instead be due to the sulphapyridine (SP) component acting as a bacteriostatic agent or to anti-inflammatory properties possessed by the intact SASP molecule [1–3]. It has been suggested that the anti-inflammatory properties of both SASP and 5-ASA may be due to their ability to inhibit production of the immunoregulatory and vasoactive prostaglandins (PGs) PGE_2 [4–7], $PGF_{2\alpha}$ [4] and PGI_2 [6]. However, conflictingly in other studies SASP has, depending on the concentration used, both enhanced and inhibited production of $PGF_{2\alpha}$ [8] and enhanced production of PGE_2 [9–11]. Similarly, 5-ASA enhanced production of PGE_2 [10] or, once again depending on the concentration used, both enhanced and inhibited production of PGI_2 [12, 13] and $PGF_{2\alpha}$ [13]. One explanation is that the effects of SASP and its metabolites on PG production *in vitro* depend on the incubation conditions, namely the concentration of drug, the PG measured and the degree of stimulation of PG production.

The aim of this study was therefore to investigate the effects of increasing concentrations of SASP and its metabolites on PG production by stimulated and non-stimulated peripheral blood mononuclear (MN) cells. MN cells were chosen because they are an accessible nucleated human tissue known to synthesize PGs, the production of which can be affected by SASP *in vitro* [10, 11]. Furthermore, the effects of SASP and 5-ASA on MN cell function *in vitro* [1, 3] are reported to be similar to those *in vivo* [2], and since MN cells take part in chronic inflammation it is possible that the effects of these drugs *in vitro* can be related to the anti-inflammatory effects of these drugs in IBD and RA patients.

MATERIALS AND METHODS

Cell separation. The effects of SASP, SP and 5-ASA on PG production were studied using cells from seven healthy male volunteers. The study of all three drugs at different concentrations, under both stimulated and non-stimulated conditions, produced a sufficiently large number of samples to permit the study of a single individual only on a single day. On each day, heparinized venous blood (50 mL) was collected from a healthy male volunteer, who had not taken any non-steroidal anti-inflammatory drugs (NSAIDs) within the previous 7 days, and centrifuged at 200 *g* for 10 min, the plasma supernatant discarded and the volume reconstituted with 1:1 (v/v) RPMI-1640 culture medium (Imperial Laboratories Ltd) (containing 24.6 mmol/L sodium bicarbonate, 2 mmol/L L-glutamine, 2×10^3 U/L benzyl penicillin, 0.140 mmol/L streptomycin sulphate) and 10 mmol/L EDTA in magnesium and calcium free phosphate-buffered saline (RPMI-PBS/EDTA) pH 7.4. EDTA was included to prevent clumping of cells during separation, washing and counting [14].

Peripheral blood MN cells were prepared using

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† Abbreviations: SASP, sulphasalazine; PG, prostaglandin; 5-ASA, 5-aminosalicylic acid; SP, sulphapyridine; IBD, inflammatory bowel disease; RA, rheumatoid arthritis; MN, mononuclear; NSAID, non-steroidal anti-inflammatory drug; RIA, radioimmunoassay; LPS, lipopolysaccharide.

aseptic reagents and conditions as described previously [15]. Ficoll-Hypaque was prepared from 7.36 g Ficoll 400 (Pharmacia, Central Milton Keynes, U.K.), 10.3 g meglumine diatrizoate, 5.15 g sodium diatrizoate made up to 100 mL in water, containing 0.1 mmol/L EDTA and 0.05 mmol/L calcium chloride. The Ficoll-Hypaque was sterilized by passing through a 0.2 μm pore size filter (Acrodisc, Gelman) and kept in the dark at 4° until required. Briefly, 10 mL cell suspension were layered onto 8 mL Ficoll-Hypaque and centrifuged at 400 g for 30 min at room temperature. The MN cells were aspirated and washed three times by sequential centrifugation and resuspension in the RPMI-PBS/EDTA solution. Total leucocyte counts and polymorphonuclear cell contamination of the MN preparations were assessed by counting an aliquot of MN cells stained with 0.1% (w/v) Methylene blue in 0.175 mol/L acetic acid. Viability was assessed by exclusion of 0.32% w/v Trypan blue in 0.154 mol/L sodium chloride by another aliquot of cells. The percentage of monocytes in the MN cell preparation was assessed using the non-specific esterase stain [16] with appropriate reagents (Technicon Instruments, NY, U.S.A.).

Preparation of drugs. Separate stock solutions of 1.1×10^{-2} mol/L SASP, 5-ASA and SP were prepared fresh each day in 0.02 mol/L sodium hydroxide. Each solution was then sterilized through a 0.2 μm pore size filter and serially diluted in sterile 0.02 mol/L sodium hydroxide to produce a concentration range of 1.1×10^{-6}–1.1×10^{-2} mol/L. Drug solutions were stored temporarily at 4° in the dark until required to prevent oxidation by light.

Incubation. Stimulated MN cell incubations containing 10 μg/mL lipopolysaccharide (LPS) or non-stimulated cultures were prepared to give 1×10^6 viable cells/mL of RPMI containing 50 μmol/L mercaptoethanol in the final incubation, which contained 0.1 mL of either an appropriate concentration of drug, giving a final concentration range for each drug of 10^{-7}–10^{-3} mol/L, or 0.1 mL of 0.02 mol/L sodium hydroxide as control. Addition of drugs or sodium hydroxide had no effect on the final pH of the incubation medium. Cells were incubated in the dark for 24 hr in a humidified atmosphere (37°, 5% CO_2) and then centrifuged at 350 g at 4° for 7 min, the supernatants stored at −20° prior to PG analysis, and an aliquot of the cells removed and tested for viability by exclusion of Trypan blue.

Assay of PG. The large number of samples from each experiment using the cells from a single individual required that a separate assay be performed for each individual studied. Samples were assayed in duplicate by radioimmunoassay (RIA) as described previously [17, 18] using commercial antisera from Advanced Magnetics Inc. (MA, U.S.A.) for the cyclo-oxygenase products PGE_2, $PGF_{2\alpha}$ and PGI_2, measured as its stable hydrolysis product 6-keto $PGF_{1\alpha}$ ($6KF_{1\alpha}$). Standards were prepared and diluted in the RPMI-1640 incubation medium to be as similar to the cell samples as possible.

Briefly, to duplicate 100-μL aliquots of standard or cell sample, 100-μL aliquots of appropriate tracer and antibody prepared in RIA buffer (0.1 mol/L phosphate buffer, pH 7.4, containing 0.154 mol/L sodium chloride, 15.4 mmol/L sodium azide and 1 g/L gelatine; BDH, Poole, U.K.) were added, the solutions mixed and left at 4° overnight. Aliquots (0.5 mL) of a suspension containing 0.5% charcoal (BDH) and 0.05% dextran (Pharmacia, Milton Keynes, U.K.) (w/v) in RIA buffer were added, the tubes centrifuged at 2000 g at 4° for 5 min, the supernatants decanted, 10 mL Opti-fluor scintillant (Canberra Packard, Pangbourne, U.K.) added, and the radioactivity counted with curve plotting of standards and calculation of unknowns performed automatically using a LKB Rackbeta 1215 counter.

Table 1. Effect of LPS stimulation of MN cells on PG production in control incubations

	pg PG produced/10^6 cells	
	Basal	LPS stimulated
PGE_2	200 (120–1101)	555 (191–1258)
$PGF_{2\alpha}$	389 (218–1324)	690 (354–1460)
$6KF_{1\alpha}$	40.2 (35.1–107)	56.3 (41.3–113)
Total PG	641 (374–2532)	1301 (535–2832)

Stimulation of cells with LPS significantly ($P < 0.001$) enhanced production of all PG measured.
Median (range), N = 7.

Reagent blanks prepared from medium, medium plus sodium hydroxide or medium plus drugs, as for the various cell incubations, gave no detectable readings in the assays. The co-efficients of intra-assay variation, calculated from the differences between duplicates [19] were 7.29% for PGE_2, 7.73% for $PGF_{2\alpha}$ and 9.83% for $6KF_{1\alpha}$. The coefficient of inter-assay variation calculated from samples assayed in each of the seven assays was 8.88% for PGE_2, 8.29% for $PGF_{2\alpha}$ and 11.68% for $6KF_{1\alpha}$.

Results for the effects of stimulation of PG production (Table 1) are derived from the mean of the three drug controls (no drug) performed in unstimulated and stimulated incubations for each subject, and expressed as the median and range for the seven subjects studied. Due to the non-normal distribution of PG production the effects of the drugs on PG production (Figs 1–3) were analysed by Wilcoxon's rank sum test for paired data, comparing the results for each concentration of drug tested with control (no drug).

Results are expressed as a percentage of the control (no drug) values and presented as mean ± SEM. As expression of the results, without regard to the absolute magnitude of PG production, might confuse an effect of the drugs on redirection of the endoperoxide intermediate from one PG to another with inhibition of PG production the results were also expressed as total PG production, derived from the sum of the three PGs measured. Where all three PGs were either inhibited or enhanced together to determine whether one PG was affected more than another ANOVA was initially performed and if a significance of $P < 0.05$ was achieved the Mann–

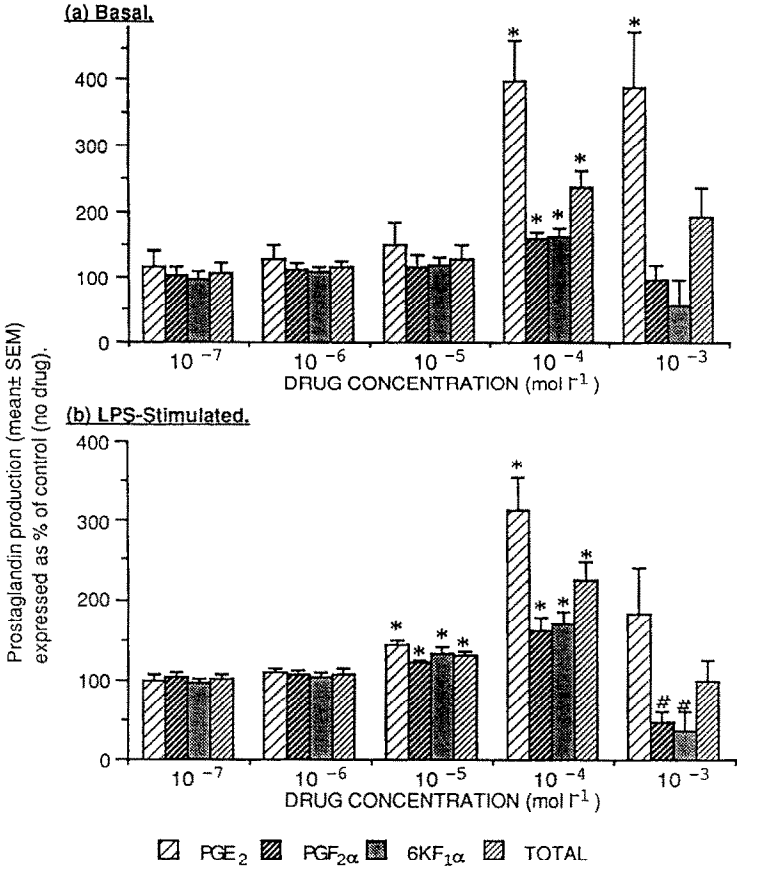

Fig. 1. Effects of SASP on (a) basal and (b) LPS-stimulated MN cell PG production. Results are expressed relative to control incubations with no drug added as means ± SEM for seven experiments performed using cells from seven individuals (*$P < 0.01$, #$P < 0.05$).

Whitney U test was performed to determine the nature of the difference.

The data for the effects of the drugs on percentage viability (Table 2) were normally distributed and thus analysed by paired *t*-test and expressed as mean ± SEM. To determine if there were any differences between the effects of the drugs on MN cell viability when more than one drug affected the viability ANOVA was initially performed and then, if a significance of $P < 0.05$ was achieved, the nature of the difference determined using an unpaired *t*-test.

All reagents used were from the Sigma Chemical Co. (Poole, U.K.), unless stated otherwise.

RESULTS

Prostaglandin production

Stimulation of MN cells in the absence of drug significantly ($P < 0.001$) increased PG production in all incubations (Table 1).

SASP significantly increased basal production of all three PGs at a concentration of 10^{-4} mol/L, but only PGE_2 at 10^{-3} mol/L (Fig. 1a). Similar results were obtained in LPS-stimulated incubations (Fig. 1b), except that a significant increase in PG production occurred at the lower concentrations of 10^{-5} and 10^{-4} mol/L. Production of $PGF_{2\alpha}$ and $6KF_{1\alpha}$ was inhibited at 10^{-3} mol/L, while production of PGE_2 was maintained but neither enhanced nor inhibited. The increase in PGE_2 production at 10^{-4} mol/L was significantly greater than that for either $PGF_{2\alpha}$ or $6KF_{2\alpha}$ in basal ($P < 0.0002$, ANOVA; $P < 0.005$, Mann–Whitney U test) and LPS-stimulated ($P < 0.001$; ANOVA: $P < 0.01$; Mann–Whitney U test) MN cell incubations but all three PGs were increased equally in LPS-stimulated incubations at 10^{-5} mol/L. Total PG production was enhanced by SASP at 10^{-4} mol/L in basal and also at 10^{-5} mol/L in LPS-stimulated incubations, a reflection of the changes in the individual PGs measured. Total PG production was not affected at 10^{-3} mol/L SASP in either basal or LPS-stimulated incubations, and thus the inhibition of some PGs observed may be due more to redirection of the endoperoxide than inhibition of the cyclooxygenase. The dose–response relationship between SASP and PG production was such that PG production increased with SASP concentration to a maximum and then declined. The effect of stimulation with LPS was to shift this relationship by an order of magnitude of SASP concentration lower.

Although 5-ASA only slightly increased non-

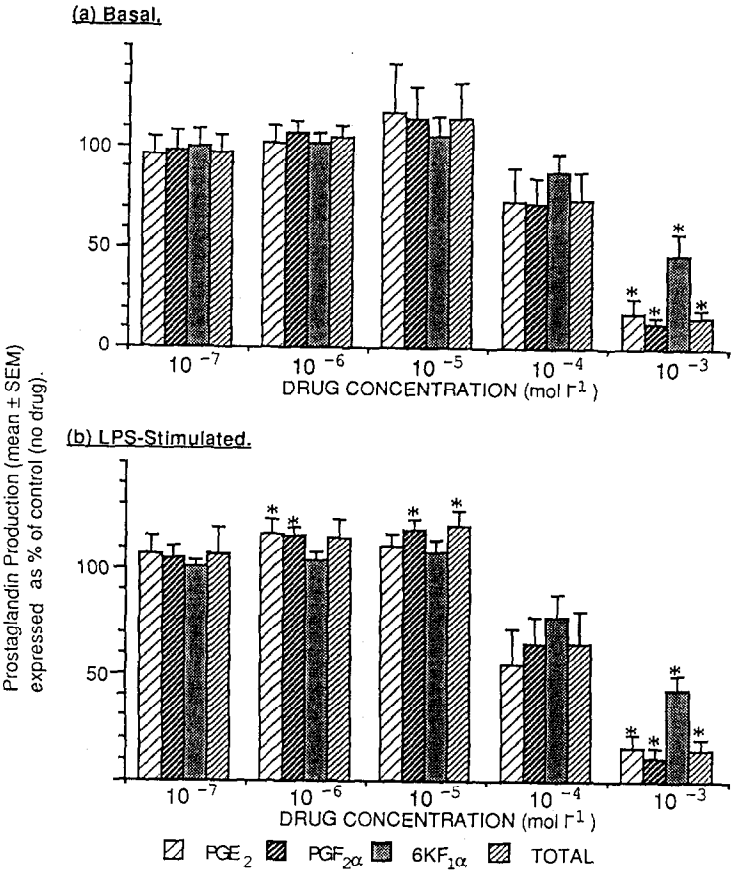

Fig. 2. Effects of 5-ASA on (a) basal and (b) LPS-stimulated MN cell PG production. Results are expressed relative to control incubations with no drug added as means ± SEM for seven experiments performed using cells from seven individuals (*$P < 0.001$).

stimulated PG production at 10^{-5} mol/L (Fig. 2a), in LPS-stimulated incubations this was significant ($P < 0.01$) even at 10^{-6} mol/L for PGE_2 and $PGF_{2\alpha}$, and at 10^{-5} mol/L for $PGF_{2\alpha}$ and total PG production (Fig. 2b). 5-ASA significantly ($P < 0.01$) inhibited both unstimulated and LPS-stimulated production of all three PGs, and hence total PG production at 10^{-3} mol/L (Fig. 2). PGE_2 and $PGF_{2\alpha}$ were inhibited equally, but both more than $6KF_{2\alpha}$ by 10^{-3} mol/L 5-ASA in both basal ($P < 0.01$, ANOVA; $P < 0.02$ and 0.02, respectively, Mann–Whitney U tests) and LPS-stimulated ($P < 0.002$, ANOVA; $p < 0.02$ and 0.005, respectively, Mann–Whitney U tests) incubations.

SP did not stimulate MN cell PG production at any concentration (Fig. 3a) and at 10^{-4} mol/L was a more effective inhibitor of non-stimulated PGE_2, $PGF_{2\alpha}$ and total PG production than 5-ASA or SASP, although the inhibition at 10^{-3} mol/L was not as great as that seen with 5-ASA. As before, LPS stimulation shifted the dose–response relationship to the left such that 10^{-5} mol/L SP inhibited PGE_2, $PGF_{2\alpha}$ and total PG production, and greater concentrations inhibited production of all three PGs. Although, in basal incubations, there was no significant difference in the degree that individual PGs were inhibited by 10^{-3} mol/L SP, in LPS-stimulated incubations SP inhibited PGE_2 and $F_{2\alpha}$ equally but more than $6KF_{2\alpha}$ at 10^{-4} mol/L ($P < 0.003$, ANOVA; $P < 0.02$ for both, Mann–Whitney U test) and at 10^{-3} mol/L ($P < 0.004$, ANOVA; $P < 0.002$ and 0.02, respectively, Mann–Whitney U test).

The individual PGs were each affected differently by the three drugs, for example, the PGs most affected by SASP were not those most affected by 5-ASA and SP. SASP (Fig. 1) enhanced PGE_2 more than $PGF_{2\alpha}$ and $6KF_{1\alpha}$ and, at high concentrations, inhibited $PGF_{2\alpha}$ and $6KF_{1\alpha}$ but not PGE_2, whereas 5-ASA (Fig. 2) and SP (Fig. 3) similarly inhibited PGE_2 and $PGF_{2\alpha}$ more than $6KF_{1\alpha}$ in most incubations where inhibition occurred.

Viability of incubated cells

The viability of cells prior to incubation was >97% (98.14 ± 0.71% mean ± SD). The incubated leucocytes contained 10.92 ± 5.5% of polymorphonuclear cells. Of the MN cells 20 ± 5.6% were monocytes. Preliminary experiments indicated that the viability of cells at 24 hr was reduced by high (10^{-3} mol/L) concentrations of SASP and 5-ASA but that stimulation with LPS did not affect

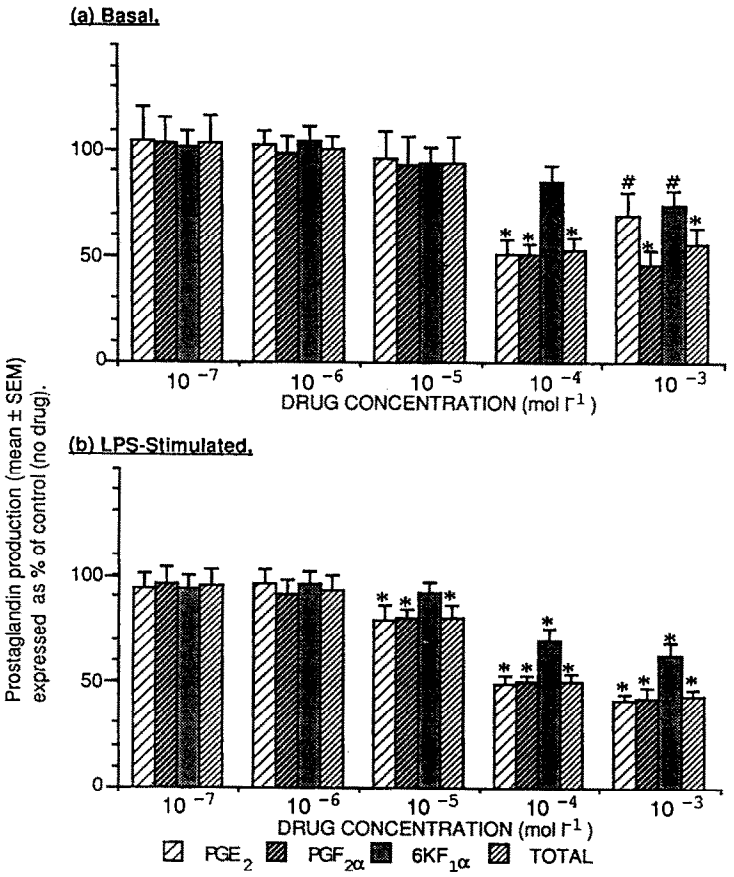

Fig. 3. Effects of SP on (a) basal and (b) LPS-stimulated MN cell PG production. Results are expressed relative to control incubations with no drug as means ± SEM for seven experiments using cells from seven individuals (*P < 0.01, #P < 0.05).

Table 2. Effects of SASP and its metabolites on MN cell viabilities after 24 hr incubation

Drug added	Control	% Viable cells for each concentration of drug (mol/L) 1×10^{-7}	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
SASP	92.9 (1.33)	89.7 (1.47)	90.0 (1.36)	90.9* (1.18)	87.0† (1.72)	66.4† (1.67)
5-ASA	94.3 (0.99)	92.3 (0.81)	94.1 (1.46)	92.4 (1.64)	90.1 (1.26)	86.1† (1.57)
SP	93.1 (0.80)	91.1 (1.30)	90.3 (1.49)	90.7 (1.25)	90.4 (0.68)	93.9 (0.75)

Means (±SEM).
Cell viability as compared to control incubations with no drug added (*P < 0.05, †P < 0.02; Wilcoxon's rank sum test).

the viability (control = 96.0 ± 2.0 vs 95.6 ± 1.5; SASP = 62.3 ± 1.20 vs 62.6 ± 4.3, N = 3; 5ASA = 81.3 ± 0.80 vs 82.3 ± 2.26, N = 2; SP = 96.5 ± 0.50 vs 93.5 ± 1.5, N = 2; mean ± SEM, basal vs LPS stimulated). Thus, the cytotoxic effects of the drugs were investigated further in parallel using the same seven subjects as for the PG studies, measuring viability at 24 hr. As stimulation with LPS had no discernible effect on viability the effects of the drugs on viability were only investigated in basal incubations. The viability of cells in the absence of drug was reduced only marginally (Table 2), but SASP at and above 10^{-5} mol/L significantly reduced MN cell viability. 5-ASA at 10^{-3} mol/L also reduced viability, although to a significantly lesser degree than SASP ($P < 0.001$, ANOVA and unpaired t-test). SP had no effect on viability.

DISCUSSION

The enhancement of PG production by SASP is in agreement with previous findings in incubations

of MN cells [10, 11, 20] and in colorectal mucosa incubated as either homogenates [7–9], microsomal fractions or whole cells [21]. In some of these studies, as in the present study, whether PG production was enhanced or inhibited depended on the concentration of SASP [7, 8] and the PG measured [8]. In another study, the concentration of the exogenous substrate (arachidonic acid) determined whether SASP enhanced or inhibited PGE_2 production [21]. Thus the ability of LPS-stimulated cells to respond to lower concentrations of SASP and its metabolites 5-ASA and SP than did unstimulated cells may be a result of higher concentrations of free arachidonic acid released by phospholipases activated by the stimulation with LPS. Incubations of colorectal tissue, either as biopsy specimens [5, 6] or cell free [4, 22] preparations, in which SASP, at similar concentrations to those used in the above studies, inhibited PG production may therefore have used proportions of SASP, substrate and enzyme that favoured inhibition only.

The small increase in PG production induced by 5-ASA is similar to that found in a separate study by us [23], but much less than that found in another study using MN cells from IBD patients [10]. The greater response in the latter study may indicate that cells from IBD patients are more sensitive to 5-ASA, possibly due to prior activation by the inflammatory process *in vivo*, perhaps from LPS-like endotoxins that pass across the inflamed mucosa. The ability of 5-ASA to enhance PG production *in vitro* at low but inhibit it at high concentrations has also been shown in colorectal tissue [12, 13], suggesting that 5-ASA may have such effects in the inflamed bowel. Other studies in which 5-ASA only inhibited PG production [5–9, 20, 22] may again have used proportions of drug, substrate and enzyme that favoured inhibition.

Previous studies have also shown that SP inhibits PGE_2 [5] and $6KF_{1\alpha}$ [6] production in rectal biopsy specimens and in bull seminal vesicle homogenates [22]. Conflictingly, in one of these studies SP had no effect at high concentrations [6], whereas in colonic mucosal homogenates SP enhanced production of some, but not all, of the PGs measured [8]. Thus, the type of tissue studied or the incubation condition used may also alter the effects of SP on PG production.

The enhancement of PG production by SASP and its metabolite 5-ASA could be due to inhibition of PG catabolism [24]. However, studies demonstrating inhibition of PG catabolism by SASP *in vitro* may have been performed under conditions where SASP denatured, rather than specifically inhibited, the enzyme involved [25]. Moreover, SASP enhances production of both PGs and their metabolites, when they have been measured together, suggesting that SASP-enhanced production is not due to inhibition of catabolism [21]. Enhanced PG production could also occur through increased availability of arachidonic acid due to SASP inhibiting the alternative, lipoxygenase, pathway. However, in a previous study increased MN cell PG production caused by SASP and 5-ASA was not associated with a change in lipoxygenase products [10].

PG production is regulated by the endogenous peroxide concentration [26], being activated by low [27] but inhibited by high levels of peroxides. Low concentrations of 5-ASA, acting as an antioxidant and reducing co-factor, can prevent free radical-mediated inactivation of cyclo-oxygenase [12, 13] as may SASP, which can also act as an antioxidant [28]. Conversely, as with high levels of other antioxidants [29], the inhibition of PG production at high concentrations of SASP and 5-ASA may be due to inhibition of the production of the stimulatory lipid peroxides [30] or to inactivation of the cyclo-oxygenase by free radical intermediates of the drug [31]. Some of the inconsistencies that exist between previous *in vitro* studies may therefore be due either to the addition of the antioxidants adrenaline and reduced glutathione [4, 7, 22], both of which affect PG production [7, 12, 13, 32], or to losses of endogenous antioxidants during preparation of the homogenates.

The effects of SASP on MN cell PGE_2 production may be related in part to the ability of SASP to inhibit mitogen-induced MN cell activation and proliferation [1–3, 11] and antibody synthesis [1]. However, co-administration of the PG inhibitor indomethacin did not affect this inhibition [3, 11], suggesting that the effects of SASP on lymphocyte proliferation are not mediated by PGs. Similarly, the effect upon PG production in the present study could also be due to SASP and the other compounds tested acting upon the MN cells through another mechanism, resulting indirectly in alterations in PG metabolism. How closely these studies on mitogen-stimulated lymphocytes in incubations performed over 72 hr can be related to the present 24-hr MN cell incubations is unknown. It has been suggested that the ability of SASP to inhibit mitogen-stimulated MN cell activation may be due to a toxic effect [33], which might also explain the lack of effect of indomethacin.

The reduced viabilities at high concentrations of SASP and 5-ASA in the present study may also be related to the reduction in PG production. However, LPS stimulation altered the response of the cells to the drugs but did not affect viability. Although other *in vitro* studies have also shown 5ASA and SASP to be toxic to MN cells at similar concentrations there are some inconsistencies both amongst these studies and between them and the present study. For example, in one study at a concentration of 10^{-4} mol/L although SASP was toxic to MN cells, in contrast to the present study, 5-ASA was not [33]; whereas in another study a concentration of 10^{-5} mol/L 5-ASA but not 10^{-4} mol/L SASP was toxic [2]. Other studies have also shown SASP, at approximately 10^{-4} mol/L, to be non-cytotoxic to MN cells *in vitro* [1–3]. Differences in incubation conditions between previous studies, for example the incubation time of 72 hr sometimes used [1–3, 33] as compared with 24 hr in the present study, may modify the toxic effects of these drugs and explain some of the differences between studies. Where the toxicity of SP has been studied the findings agree with those in the present study, namely that SP at 10^{-4} mol/L is not toxic to MN cells *in vitro* [2, 33].

Whether SASP or 5-ASA inhibits or enhances MN cell PG production *in vitro* thus depends on the

PG measured, whether cells are stimulated, and the nature and concentration of the drug. Whereas the concentrations of these drugs in the bowel lumen (*ca.* 10^{-4}–10^{-2} mol/L) could favour either enhancement or inhibition of PG production, they are probably much lower in the mucosa where they may enhance PG production. Inhibitors of PGs, such as NSAIDs, are ineffective or exacerbate IBD, whereas some PGs [34] or their analogues [35] are anti-inflammatory in the colon; thus, SASP and 5-ASA could act therapeutically by enhancing protective PG production. Reductions in PG production associated previously *in vivo* with treatment may have been due rather to alterations in the number of PG-producing leucocytes in the inflamed bowel [36, 37] and changes in permeability of the bowel to PGs than to a direct effect on PG synthesis. The greater effects of SASP than of 5-ASA on PG production and viability, and the differences in the PGs affected by each drug suggest that SASP possesses properties different to those of 5-ASA.

However, whether the conditions in the mucosa are reproduced accurately in cell and tissue incubations is unknown and thus such *in vitro* studies should be extrapolated to the therapeutic mechanisms *in vivo* with caution.

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