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INHIBITION OF NEUTROPHIL DEGRANULATION AND SUPEROXIDE PRODUCTION BY SULFASALAZINE

COMPARISON WITH 5-AMINOSALICYLIC ACID, SULFAPYRIDINE AND OLSALAZINE

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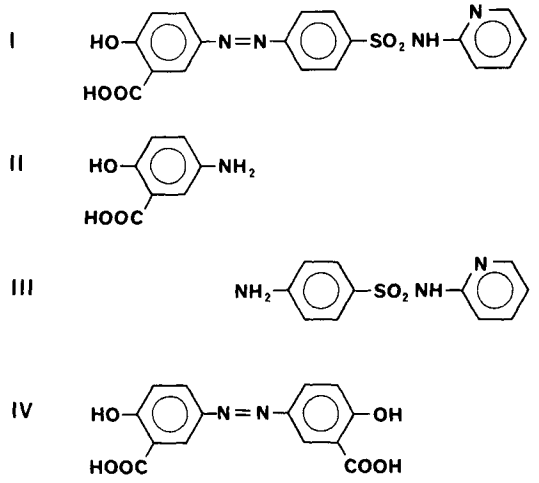
Abstract—Sulfasalazine is a potent inhibitor of superoxide production and granule enzyme release by stimulated neutrophils, and modulation of these responses may contribute to its anti-inflammatory properties. It is a composite drug consisting of 5-aminosalicylic acid and sulfapyridine joined through an azo linkage. To investigate which functional groups on the molecule are active against neutrophil responses, 5-aminosalicylic acid, sulfapyridine and olsalazine were added to cells stimulated with fMet-Leu-Phe or immune complexes. The inhibitory effects of sulfasalazine on superoxide production, degranulation and neutrophil-mediated collagen degradation were closely mimicked by olsalazine, with the other two compounds having little effect on either function. Thus the azo link appears to be the important structural feature of sulfasalazine that affects neutrophil responses. This suggests that sulfasalazine could be anti-inflammatory in its own right rather than just acting as a source of 5-aminosalicylic acid. Our findings are also a favourable indication for olsalazine (Dipentum), which is currently under trial as an anti-inflammatory agent.

Sulfasalazine (Salazopyrin) is widely used in the treatment of ulcerative colitis [1–3], and it appears to be beneficial in some cases of rheumatoid arthritis [4, 5]. It is a composite molecule consisting of 5-aminosalicylic acid (5-ASA) linked to sulfapyridine by an azo linkage. The rationale in its original design was to combine an antibiotic with an anti-inflammatory agent [1], and its effectiveness in inflammatory bowel disease relates to its being poorly absorbed, then hydrolysed by colon bacteria to release 5-ASA [6, 7]. However, it seems likely that the anti-inflammatory activity of sulfasalazine is not solely due to 5-ASA [4, 5, 8, 9].

As well as inhibiting prostaglandin synthesis [9, 10], sulfasalazine modulates a variety of neutrophil functions [8, 11–15]. It strongly inhibits superoxide production and degranulation of neutrophils stimulated with fMet-Leu-Phe [12, 13], opsonized zymosan [8] or immune complexes [13], although with the latter there is a higher concentration requirement. It also inhibits neutrophil-mediated collagen degradation [14] in an *in vitro* model system in which immune complexes are embedded in a glomerular basement membrane matrix [16].

We have investigated which functional groups on the sulfasalazine molecule (I) are responsible for its effects on neutrophils, by comparing the constituent molecules, 5-ASA (II), sulfapyridine (III) and olsalazine, a 5-ASA dimer linked with an azo bond (IV). Olsalazine (Dipentum) is currently undergoing clinical trial for treatment of inflammatory bowel disease [17].



MATERIALS AND METHODS

Human neutrophils were prepared from heparinized blood by the method of Böyum [18] and resuspended in 5 mM phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mg/ml glucose. Methodology for the preparation of albumin–anti-albumin immune complexes and

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† Abbreviations used: 5-ASA, 5-aminosalicylic acid; IgG, immunoglobulin G.

aggregates of purified immunoglobulin (Ig) G, for stimulation of the neutrophils with fMet-Leu-Phe or immune complexes, and for measuring superoxide production (as cytochrome *c* reduction) and release of myeloperoxidase, β-glucuronidase and lysozyme are described in refs 13 and 16.

Glomerular basement membrane was prepared from normal human kidney and impregnated with immune complexes [16]. The effects of the drugs on neutrophil oxygen uptake, granule enzyme release and basement membrane degradation, measured as hydroxyproline solubilization [16] were determined as described previously [14].

The drugs were tested for interference in each of the assays. 5-ASA reduced cytochrome *c* directly and could not be used in the superoxide assay. The other compounds were tested for their effect on cytochrome *c* reduction by a xanthine oxidase superoxide generating system. Although both sulfasalazine and olsalazine inhibited in this system, they inhibited urate production by the xanthine oxidase to a similar extent. Their effect, therefore, appeared to be on the enzyme, and we saw no evidence that they were affecting detection of superoxide. 5-ASA interfered in the myeloperoxidase assay, and sulfasalazine and olsalazine at high concentrations interfered in the detection of β-glucuronidase. Otherwise the drugs caused less than 15% inhibition in any assay. β-Glucuronidase was measured as the azurophil marker except with >250 μM sulfasalazine or olsalazine.

Sulfasalazine, sulfapyridine, 5-ASA and olsalazine were a gift from Mr Peter Chapman, Pharmacia, North Ryde, Australia. Other biochemicals were obtained from the Sigma Chemical Co., St Louis, MO.

RESULTS

Superoxide production and degranulation of neutrophils stimulated with fMet-Leu-Phe and immune complex

In agreement with previous observations [13], sulfasalazine inhibited superoxide production and release of granule enzymes from neutrophils stimulated with either fMet-Leu-Phe or immune complexes (Fig. 1a). A 10-fold lower drug concentration was required to inhibit responses to fMet-Leu-Phe, possibly because sulfasalazine can compete for its surface receptor [12].

Sulfapyridine did not affect granule enzyme release with either stimulus, and gave slight inhibition of superoxide production only with fMet-Leu-Phe (Fig. 1b). 5-ASA gave no inhibition of enzyme release (Fig. 1c). Although it interfered in the superoxide assay, 1000 μM 5-ASA gave no inhibition of O_2 uptake by IgG-stimulated cells (not shown). Olsalazine, however, mimicked the behaviour of sulfasalazine, inhibiting superoxide production and degranulation with I_{50}s of about 20 and 50 μM respectively with fMet-Leu-Phe, and at higher drug concentrations with immune complexes (Fig. 1d).

Basement membrane degradation

Immune complexes associated with isolated basement membrane stimulate neutrophils to adhere, release superoxide and granule enzymes, and degrade the membrane proteins [16]. Degradation can be measured as hydroxyproline solubilization, and is due to released neutral proteinases, mainly elastase and gelatinase [19]. Anti-inflammatory drugs that inhibit neutrophil responses to immune complexes, including sulfasalazine, have been shown to inhibit degradation [14]. Using this model of neutrophil-mediated inflammatory tissue damage, olsalazine again mimicked sulfasalazine, and inhibited basement membrane degradation, while sulfapyridine was ineffective (Fig. 2). 5-ASA gave slight inhibition (81 ± 8% of control degradation at 250–1000 μM).

Granule enzyme release and oxygen uptake were also inhibited by sulfasalazine and olsalazine in the basement membrane system (Fig. 2). The only notable difference between the two compounds is that sulfasalazine but not olsalazine inhibited lysozyme release. Most of the basement membrane collagen degradation in this system is due to elastase [19]. It is likely, therefore, that inhibition of elastase release from the azurophil granules is primarily responsible for the decreased degradation. Although sulfapyridine gave some inhibition of release of the azurophil marker myeloperoxidase, this was not reflected in decreased basement membrane degradation.

DISCUSSION

This study has demonstrated that sulfasalazine and olsalazine are equally potent inhibitors of superoxide production and degranulation of human neutrophils. Both agents had similar effects on neutrophils stimulated either with fMet-Leu-Phe, or with immune complexes, and both inhibited neutrophil-mediated basement membrane degradation. Sulfasalazine is generally thought of as a vehicle for delivering 5-ASA (and possibly sulfapyridine) to sites of inflammation [1, 2, 5, 17], yet both these constituents were considerably less active against neutrophil responses. One similar study of sulfapyridine and 5-ASA (but not olsalazine) has been reported [8]. This agrees with our negative findings with 5-ASA, but (with opsonized zymosan-stimulated neutrophils) inhibition of superoxide production by sulfapyridine was greater than in our study. Our results with the three stimuli are consistent, and indicate that the azo linking group is an important feature for the inhibition of neutrophil responses. They also imply that sulfasalazine may be anti-inflammatory in its own right.

One explanation for the anti-inflammatory properties of sulfasalazine is inhibition of cyclo-oxygenase by 5-ASA [9, 10]. However, other cyclo-oxygenase inhibitors are generally unsatisfactory for treating ulcerative colitis [9], and it seems likely that there are other contributing effects. One could well be inhibition of neutrophil stimulus responses. This would reduce not only the release oxidants and hydrolytic enzymes, but also of mediators such as leukotrienes that amplify the inflammatory process. Inhibition of leukotriene synthesis in isolated neutrophils [11] and in colonic mucosa by sulfasalazine has been observed [20, 21]. Extrapolation of *in vitro* findings to therapeutic effects must be made cau-

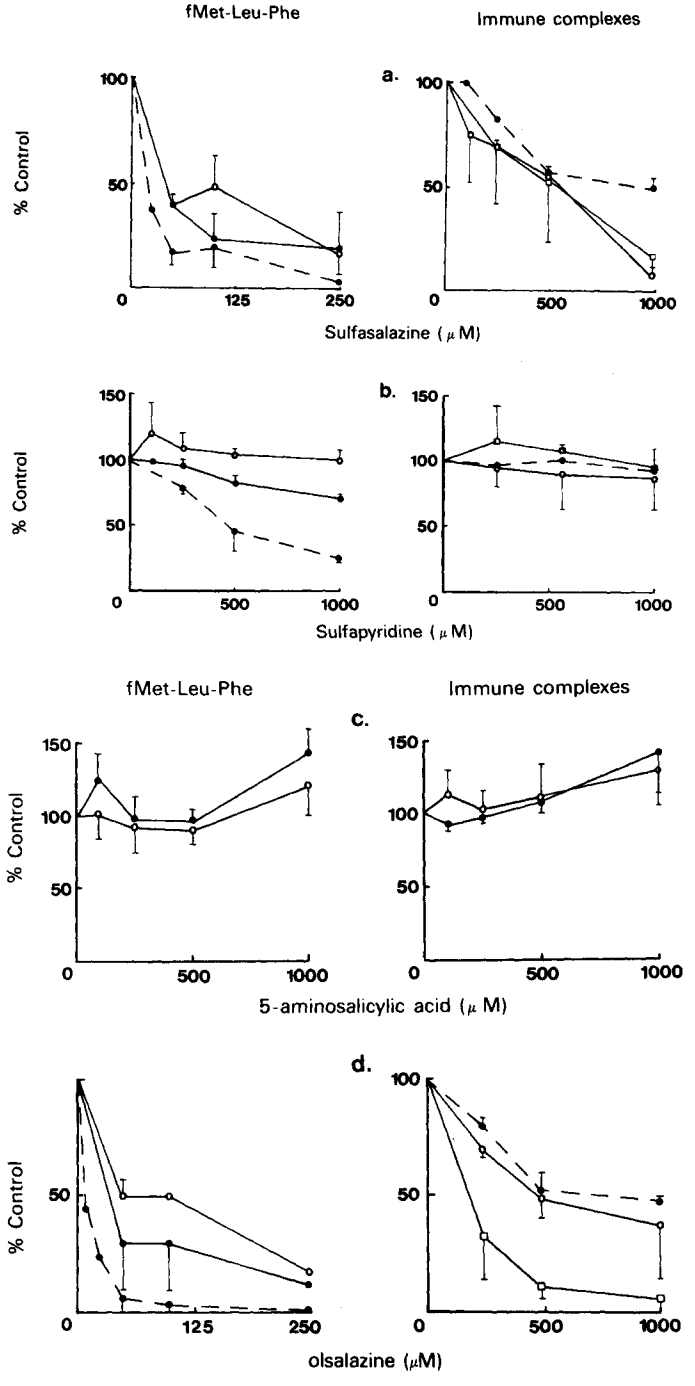

Fig. 1. Effects of (a) sulfasalazine, (b) sulfapyridine, (c) 5-aminosalicylic acid and (d) olsalazine on superoxide production and granule enzyme release by neutrophils stimulated with fMet-Leu-Phe (left hand panels) or immune complexes (right hand panels). Superoxide production (●---●): Either 10^{-7}M fMet-Leu-Phe or albumin/anti-albumin complexes (125 μg albumin/ml) were added to 5×10^6 neutrophils/ml and 100 μM cytochrome *c*, which had been pre-incubated with the drug and cytochalasin B (5 μg/ml) for 5 min [13]. Cytochrome c reduction was measured continuously and maximum rates (over the first 1–3 min) were determined. Granule enzymes: Neutrophils were stimulated as above, except 1 mg/ml aggregated IgG with 10^7 neutrophils/ml was the immune stimulus [13]. Supernatants were separated after 15 min and analysed for myeloperoxidase (□——□), β-glucuronidase (●——●) and lysozyme (○——○). Results (means ± SD for 2–7 observations) are expressed relative to control values for the same cell preparation with no drug added. For fMet-Leu-Phe and immune complexes respectively mean values for release per 10^6 cells were superoxide, 1.80 ± 0.91 and 1.48 ± 0.88 nmol/min; lysozyme ΔA_{450}/min = 0.046 ± 0.016 and 0.019 ± 0.005; β-glucuronidase, 24 ± 13 and 5.6 ± 2.4 μg phenolphthalein released/24 hr; and myeloperoxidase, ΔA_{440}/min = 0.40 ± 0.19 (immune complexes).

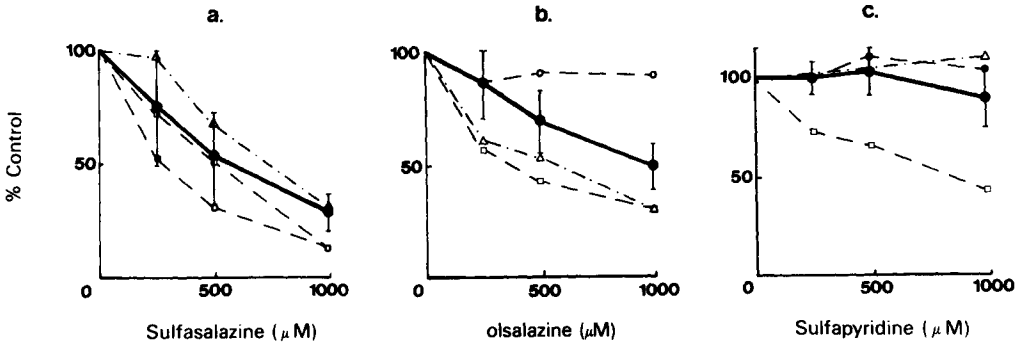

Fig. 2. Effects of (a) sulfasalazine, (b) olsalazine and (c) sulfapyridine on oxygen uptake, degranulation and collagen digestion by neutrophils added to basement membrane containing IgG aggregates. Neutrophils (10^7) in 1 ml were added to basement membrane (2 mg) with or without pre-incubation with drug. Oxygen uptake was measured directly and initial linear rates (over the first 5 min) were determined. Enzyme release and hydroxyproline solubilization were measured after 2 hr [16]. Results are expressed relative to control values with no added drug, obtained with the same cells and basement membrane preparation. Means ± SD of 3–6 observations are shown for hydroxyproline solubilization. Mean values only (from a similar number of experiments) of the other parameters are shown. Control values (means ± SD) were: hydroxyproline solubilized, 1.26 ± 0.65 μg; lysozyme release, ΔA_{450}/min = 0.022 ± 0.008; myeloperoxidase ΔA/min = 0.27 ± 0.14; O_2 uptake (maximum rate) 2.2 ± 0.7 nmol/min, all per 10^6 cells. ●——●, Hydroxyproline solubilized; ○---○, lysozyme; □---□, myeloperoxidase; △- -△, O_2 uptake.

tiously, especially in relation to dose response. However, our observed effects of sulfasalazine and olsalazine, even with immune complexes, were at concentrations similar or below those used in other *in vitro* studies [8, 11, 20, 21]. With normal 2 g daily doses [2, 4], and evidence that sulfasalazine collects in collagen-rich tissues including the intestinal wall and synovial fluid [22] drug requirements to inhibit neutrophil responses may not be excessive.

Our findings suggest that olsalazine (Dipentum) should retain the anti-inflammatory properties of sulfasalazine against neutrophil responses, without the undesirable side effects due to sulfapyridine release [5, 17]. This is a favourable indication for its potential clinical usefulness.

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REFERENCES

1. N. Svartz, *Acta med. Scand.* **110**, 577 (1942).
2. U. Klotz, K. Maier, C. Fischer and K. Heinkel, *N. Engl. J. Med.* **303**, 1499 (1980).
3. A. Dissanayake and S. C. Truelove, *Gut* **14**, 923 (1973).
4. B. McConkey, R. S. Amos, S. Durham, P. J. G. Forster, S. Hubball and L. Walsh, *Br. Med. J.* **280**, 442 (1980).
5. V. C. Neumann and K. A. Grindulis, *J. Roy. Soc. Med.* **77**, 171 (1984).
6. H. Schroder and D. E. S. Campbell, *Clin. Pharmac. Ther.* **13**, 539 (1972).
7. A. K. Azad, H. H. Johnston and S. C. Truelove, *Gut* **16**, 832 (1975).
8. L. Molin and O. Stendahl, *Acta med. scand.* **206**, 451 (1979).
9. M. Donowitz, *Gastroenterology* **88**, 580 (1985).
10. H. O. J. Collier, A. A. Francis, W. J. McDonald-Gibson and S. A. Saeed, *Prostaglandins* **11**, 219 (1976).
11. W. F. Stenson and E. Lobos, *J. clin. Invest.* **69**, 494 (1982).
12. W. F. Stenson, J. Mehta and I. Spilberg, *Biochem. Pharmac.* **33**, 407 (1984).
13. T. M. Neal, M. C. M. Vissers and C. C. Winterbourn, *Biochem. Pharmac.* **36**, 2511 (1987).
14. T. M. Neal, M. C. M. Vissers and C. C. Winterbourn, *Arthritis Rheum.* in press.
15. J. M. Rhodes, T. C. Bartholomew and D. P. Jewell, *Gut* **22**, 642 (1981).
16. M. C. M. Vissers, C. C. Winterbourn and J. S. Hunt, *Biochim. biophys. Acta* **804**, 154 (1984).
17. H. Koch, *Pharmacy Int.* **6**, 111 (1985).
18. A. Böyum, *Scand. J. clin. Lab. Invest.* **21** (Suppl. 97), 77 (1968).
19. M. C. M. Vissers and C. C. Winterbourn. *Biochim. biophys. Acta* **868**, 86 (1986).
20. S. Pinchas and W. F. Stenson, *Gastroenterology* **86**, 453 (1984).
21. C. J. Hawkey, N. K. Boughton-Smith and B. J. R. Whittle, *Digestive Dis. Science* **30**, 1161 (1985).
22. A. Hangren, E. Hansson, N. Svartz and S. Ullberg, *Acta med. scand.* **173**, 61 (1963).