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The Effect of Some Anti-inflammatory Agents on Elastase Release From Neutrophils In-vitro

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Abstract—In view of the potential role of released polymorphonuclear leucocyte elastase in causing tissue damage, the effect of commonly used anti-inflammatory drugs on elastase release from neutrophils has been studied in-vitro. Elastase release from neutrophils exposed to the synthetic bacterial cell wall peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (10^{-6} M) was quantitated using a radiometric immunoassay and a functional assay of elastase. Prednisolone and non-steroidal anti-inflammatory drugs inhibited elastase release at concentrations from 0·1 mM–0·1 nM. No inhibition by sulphosalicylic acid, D-penicillamine or chloroquine sulphate was observed. The clinical relevance of these findings is discussed.

Polymorphonuclear leucocytes (PMNL) play a central role in acute inflammatory processes. Their protective function is based partly on their phagocytic capacity and partly on their ability to release certain enzymes; this release itself is intimately connected with the potential for causing tissue damage (Cohen et al 1983).

The major enzymes of importance are elastase (Barrett 1981a; Adeyemi & Hodgson 1988), cathepsin G (Barrett 1981b) and collagenase (Gleisner 1979).

Among the major neutrophil enzymes, elastase is the only enzyme that can degrade all the major classes of connective tissue. The importance of elastase is underlined by the clinical evidence that patients who are deficient in its major inhibitor, α_1-proteinase inhibitor (α_1-PI), have an early onset of pulmonary emphysema (Eriksson 1965). This is attributed to the uninhibited action of human leucocyte elastase (HLE) on connective tissue in the lung.

We have recently shown that HLE-α_1-PI complex levels are elevated in patients with inflammatory bowel disease (Adeyemi et al 1985) and in patients with rheumatoid arthritis (Adeyemi et al 1986). Because of the general interest in the tissue degradative potential of HLE, we have studied the effect of commonly used anti-inflammatory drugs on elastase secretion in-vitro.

Materials and Methods

Chemicals and drugs

Prednisolone, acetylsalicylic acid, 4-aminosalicylic acid, *N*-formyl-L-methionyl-L-phenylalanine (FMP) and low molecular weight standard packs (Sigma Chemicals Ltd, UK). D-Penicillamine, cytochalasin B and 5-aminosalicylic acid (Aldrich Chemicals Ltd, UK). *N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (UCB, 68 Rue Berkendael, B-1060 Bruxelles, Belgium). Suc-Ala-Ala-Val-pNa (Cambridge Research Biochemicals, Cambridge, UK). Sulphosalicylic acid, *p*-nitroanalide (BDH Chemicals Ltd, Poole, UK). Hydroxyethyl starch 6% (Fresenius AG, Bad Homburg, West Germany). Cyanogen bromide activated Sepharose 4B (Pharmacia Ltd, UK). Dialysis tubing (Scientific Instrument Centre, London, UK). Microtitre well plates (Dynatech Lab Inc., Aldexandria, Virginia, USA). Microfilters (Flow Labs Ltd, UK).

The following drugs were obtained as gifts: indomethacin in DMSO (Merck Sharp & Dohme Ltd, UK), chloroquine phosphate (Glaxo Labs Ltd, UK) and aprotinin (Dr Friedrick Schumann, Bayer AG, Wuppertal, West Germany).

Buffer solutions

Tris buffered saline pH 7·4, also referred to as washing solution in the text, containing 1 mg mL^{-1} D-glucose was used to wash the cells and as drug diluent. The secretion buffer pH 7·4 contained in addition to the above 1 mM $CaCl_2$, 0·5 mM $MgCl_2$, 0·4 mM $MgSO_4$ and 1 mg mL^{-1} bovine serum albumin.

Elastase assay buffer pH 7·4 contained 10 mM HEPES, 0·1% Brij 35 and 0·01% sodium azide. Radioimmunometric assay buffer solution was phosphate buffered saline pH 7·4, containing 20 units mL^{-1} aprotinin, 2 mM EDTA, 0·1% BSA, 0·02% sodium azide.

Radioiodination of protein A

Protein 1 mg was iodinated with 1 μCi $Na^{125}I$ (Amersham International Ltd, UK) per μg protein A (Sigma Chemicals Ltd, UK) by the method of Fraker & Speck (1978). Trichloroacetate-precipitable protein-bound radioiodine was approximately 75%.

Isolation of neutrophils

Neutrophils were isolated using the hydroxyethyl starch sedimentation technique. Four mL each of 6% hydroxyethyl starch and 100 mM EDTA was added to 40 mL venous blood, obtained from six healthy volunteers (3M, 3F, mean age 30·1 years) with no known history of chronic inflammation and who were not on any anti-inflammatory drugs. The blood was allowed to settle at room temperature (20°C) for 60 min. Ten mL portions of the supernatant were underlayered with 10 mL of a solution containing 9% ficoll (Sigma Chemicals Ltd, UK) and 34% Hypaque (Sterling Research, Guildford UK) and centrifuged at 300 *g* for 30 min at room temperature. The mononuclear cell layer at the water-ficoll-hypaque interface was carefully aspirated, as was the platelet-rich supernatant. The pellet was suspended in 2 mL washing solution and the erythrocytes were lysed with chilled hypoto-

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nic phosphate buffered saline (60 mosm kg^{-1}), by mixing gently for 60 s. The osmolality was restored with hypertonic phosphate buffered saline (ca. 3000 mosm kg^{-1}). The cells were harvested by centrifugation at 150 *g* for 10 min at 4°C.

The pellet was resuspended in 2 mL washing solution and the lysis step repeated twice. The cells were counted manually in a Neubauer chamber (Dacie & Lewis 1975). The yield was ca 10^8 cells (99% neutrophils). After 2 h at room temperature, viability as tested by the trypan blue exclusion test was 100%.

Elastase secretion assay

In brief, 4 μg of cytochalasin B in 40 μL was added to $2{\cdot}5 \times 10^6$ neutrophils in 760 μL for 5 min at 37°C. To measure the inhibitory effects of drugs, 100 μL of the appropriate drug dilution was added for a further 5 min. FMLP (10^{-5} M, 100 μL) was then added—optimum concentration of FMLP 10^{-6} M which produced maximum elastase release, within 2 min was defined in preliminary experiments. The incubation was continued for 25 min. Supernatants were separated by centrifugation at 1000 *g* for 5 min at 4°C.

As indomethacin was dissolved in DMSO, diluted 10-fold to yield 10^{-4} M, control incubations included 1 and 10% DMSO. Positive controls for elastase secretion contained cytochalasin B but no added drug. Negative controls did not include FMLP.

The enzyme activity in the supernatants was determined by adding 400 μL supernatant to 400 μL elastase assay buffer, 100 μL substrate Suc-Ala-Ala-Val-pNa and incubating for 25 min at 37°C. The reaction was stopped by adding 100 μL soy bean trypsin inhibitor (BDH Chemicals, Poole, UK) (1 mg mL^{-1}) and the absorbance values determined at 405 nm. The inhibitory capacity (IC) of each agent was expressed as the percentage decrease of enzyme activity from the positive control after subtracting the non-specific activity of the negative control from each reading.

Radioimmunometric assay (RIMA)

Samples, radiolabelled protein A and antiserum were diluted in RIMA buffer. In a typical radiometric inhibition assay, the protein in 100 μL of each supernatant was adsorbed onto poly-lysine A/glutaraldehyde pre-coated wells at 37°C for 1 h. The plate was washed twice with phosphate buffered saline containing 0·05% (v/v) Tween 20. After the washing step, 100 μL each of antiserum (1:1000 dilution) and radiolabelled protein A (1:500) was added in successive steps, using 30 min incubation periods at 37°C in a wet chamber. The wells were washed twice, and the radioactivity was determined with a gamma-counter.

The inhibitory capacity (IC) was calculated as the percentage decrease of radioactivity from the positive control.

Results

The effect of prednisolone on elastase release from PMNL at different drug concentrations, detected using the functional assay with Suc-Ala-Ala-Val-pNa as substrate, is shown in Fig. 1. The derived in-vitro concentration which would cause 50% inhibition (IC50) was $10^{-7{\cdot}7}$ M. At 10^{-4} M, the mean inhibition by prednisolone was 92% ± 2·6 s.e.m.

Similarly indomethacin inhibited neutrophil elastase release in the functional assay (as above) at concentrations from 10^{-14} to 10^{-4} M (Fig. 2) with a calculated IC50 of $10^{-7{\cdot}8}$ M. Indomethacin had a mean inhibition value of 87% ± 4·5 s.e.m. at 10^{-4} M.

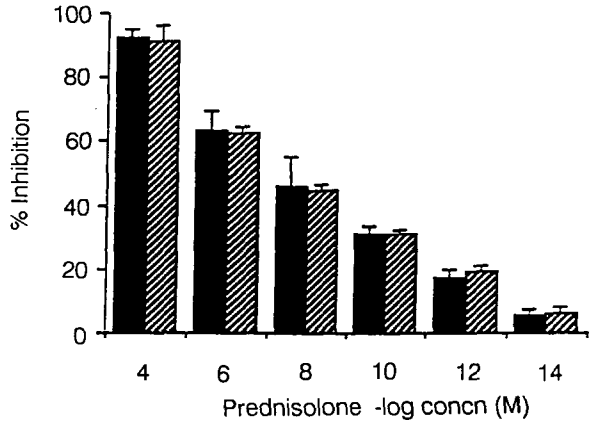

FIG. 1. Inhibition of neutrophil elastase release, expressed as percentage of positive control by various concentrations of prednisolone with a functional assay (dark bars) and a radioimmunometric assay (hatched bars). Each bar represents the mean plus s.e.m. in six individuals.

To confirm that these drug effects did not reflect functional inhibition of released enzyme, the actual quantity of elastase released into the supernatant was measured radioimmunometrically (Figs 1, 2). No statistically significant difference was found between results obtained with the two methods for either prednisolone ($P = 0{\cdot}95$ Mann-Whitney; $P = 0{\cdot}99$ Student's *t*-test) or indomethacin ($P = 0{\cdot}95$ Mann-Whitney; $P = 1{\cdot}0$ Student's *t*-test).

RIMA was used to assess the inhibitory capacity of acetylsalicylic acid (ASA), 5-amino-ASA, 4-amino-ASA, sulphosalicylic acid, chloroquine, cimetidine and D-penicillamine. Sulphosalicylic acid, chloroquine, cimetidine and D-penicillamine showed no appreciable inhibition of elastase release between 10^{-12} and 10^{-4} molar concentrations. However, sulphosalicylic acid demonstrated 95% inhibition of

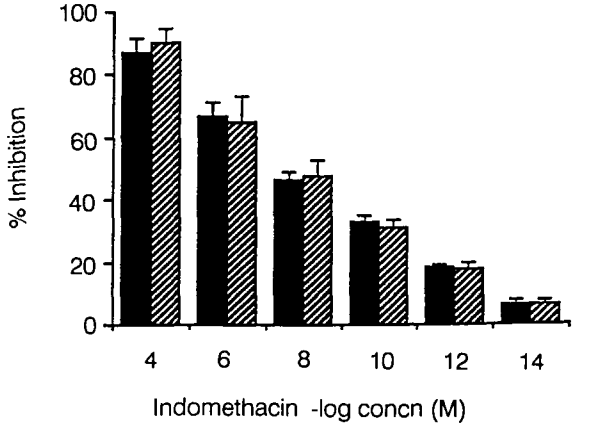

FIG. 2. Inhibition of neutrophil elastase release, expressed as percentage of positive control by various concentrations of indomethacin using a functional assay (dark bars) and a radioimmunometric assay (hatched bars). Each bar represents the mean plus s.e.m. in six individuals.

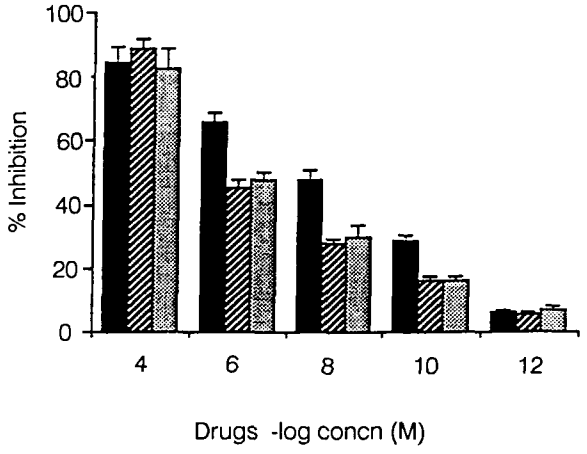

FIG. 3. Inhibition of neutrophil elastase release expressed as percentage of positive control by various concentrations of: 4-aminosalicylic acid (dotted bars); acetylsalicylic acid (aspirin) (dark bars); 5-aminosalicylic acid (hatched bars) using a radioimmunometric assay. Each point represents the mean of measurements in five different individuals.

elastase release at 10^{-2} M, below which inhibition was negligible. On the other hand inhibition was observed in five volunteers at concentrations of ASA ranging from 10^{-12} to 10^{-4} M with an IC50 value of $10^{-7.9}$ M. Inhibitory capacity values of 84·6% ± 4·6 s.e.m. and 48% ± 2·7 s.e.m. were recorded at 10^{-4} and 10^{-8} M, respectively (Fig. 3).

Fig. 3 demonstrates the inhibition of neutrophil elastase release by 5-amino-ASA and 4-amino-ASA with each having an IC50 value of 10^{-6} M. At 10^{-4} M, 5-amino-ASA and 4-amino-ASA had mean IC values of 89% ± 3·0 s.e.m. and 82·5% ± 6·6 s.e.m., respectively. At 10^{-8} M, their respective mean IC values were 28·2% ± 1·3 s.e.m. and 30% ± 3·5 s.e.m.

Discussion

The study of neutrophil elastase release in-vitro has been aided by the availability of a suitable spectrophotometric substrate such as Suc-Ala-Ala-Val-pNa which is specific for neutrophil elastase (Wenzel et al 1980). Neutrophil elastase secretion in response to FMLP forms the basis of the technique used in this study.

We used this peptide at a dose of 10^{-6} M, which in this system produced maximum enzyme release. Maximum elastase activity was detected within the first 2 min. This finding agrees with the result obtained by Sklar et al (1982) who detected maximum elastase activity within one min of addition of FMLP.

The mechanism by which FMLP causes neutrophil degranulation is complex, but this peptide has been shown to stimulate, in neutrophils, the synthesis and release of eicosanoids, including prostaglandin E_2, prostaglandin $F_{2\alpha}$, thromboxane and products of the lipoxygenase pathway (Nast & LeDuc 1988). Recent findings suggest that FMLP activates protein kinase C (Itami et al 1987). Several mechanisms might be proposed to explain the effect of prednisolone, indomethacin, ASA and ASA derivatives on neutrophil elastase secretion in-vitro. Inhibition of elastase release might involve arachidonic acid metabolism (Nast & LeDuc 1988). It is known that prednisolone inhibits phospholipase A2 (PLA2) (Schumert et al 1988), which has been described in neutrophils (Victor et al 1981). At high concentrations, indomethacin would inhibit both pathways of arachidonic acid metabolism (Siegel et al 1979). ASA inhibits cyclo-oxygenase by direct acetylation (Flower 1974). 5-Amino-ASA, used extensively in treating inflammatory bowel disease, also affects the cyclo-oxygenase pathway in human colonic tissue (Peskar et al 1987) and inhibits in a dose-dependent fashion the release from neutrophils of leukotriene B4 (LTB4) and 5-hydroxyeicosatetranoic acid (5-HETE), both products of the lipoxygenase pathway (Nielsen et al 1987). Drugs with no demonstrated effects on arachidonic acid metabolism such as cimetidine, chloroquine and D-penicillamine had no effect on neutrophil elastase release.

Alternatively, these drugs might modulate the activation of protein kinase C-dependent system in granulocytes. The length of time required for this effect to reach a maximum was 15 min (Itami et al 1987), which might also explain why inhibition of elastase release was possible within such a short preincubation period (5 min in this assay).

A similarly short in-vitro preincubation period of 10 min was reported by Lelievre et al (1988) who demonstrated inhibition of the cyclo-oxygenase pathway in platelets by steroids and non-steroidal anti-inflammatory drugs.

The concentration of prednisolone needed for half-maximal reduction of elastase release (IC50 = 10^{-8} M = 3·6 ng mL^{-1}) in this study is in the lower range of plasma levels obtained when 20 mg prednisolone was injected intravenously (Lennard-Jones 1984). Much higher plasma prednisolone levels are to be expected, when higher doses of prednisolone are administered in life-threatening conditions. In addition higher tissue prednisolone concentrations are expected, when enemas are administered in the treatment of ulcerative colitis (Klotz & Maier 1987).

At therapeutic doses, the indomethacin plasma concentration was found to be 0·3–0·8 μM by Rane et al (1978). At this concentration (ca 10^{-6} M) indomethacin demonstrated ca 50% inhibition, confirming that the observations reported here are relevant to the drug concentration achieved in clinical practice.

Salicylic acid derivatives showed similarly high IC values (Fig. 3), except sulphosalicylic acid which inhibited neutrophil elastase release at 10^{-2} M only. This is important as ASA and its derivatives are widely used to treat various forms of active connective tissue (Bardare et al 1978) and inflammatory bowel disease (Klotz & Maier 1987). The therapeutic salicylate level was found to be 1·1–2·2 mM (Bardare et al 1978) in plasma, a concentration much higher than 10^{-4} M, the highest concentration of 5-amino-ASA used in this study. The concentration of 5-amino-ASA required to cause a half-maximal inhibition of elastase release (IC50 = 10^{-6} M = 0·153 μg mL^{-1}) is within the range of 5-amino-ASA steady state plasma levels obtained when 250 mg–1 g thrice daily (Klotz & Maier 1987) was given to patients. A much higher concentration may occur in tissues when high dose 5-amino-ASA enemas are administered in ulcerative colitis (Klotz & Maier 1987).

Despite the shortcomings of an in-vitro study, this work suggests that inhibition of elastase release is one mechanism of action of anti-inflammatory drugs, and demonstrates that this effect is not due to functional inhibition of elastase activity.

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