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INHIBITION BY NONSTEROIDAL ANTI-INFLAMMATORY DRUGS OF SUPEROXIDE PRODUCTION AND GRANULE ENZYME RELEASE BY POLYMORPHONUCLEAR LEUKOCYTES STIMULATED WITH IMMUNE COMPLEXES OR FORMYL-METHIONYL-LEUCYL-PHENYLALANINE

TERESA M. NEAL, MARGRET C. M. VISSERS and CHRISTINE C. WINTERBOURN*
Pathology Department, Christchurch School of Medicine, Christchurch Hospital, Christchurch, New Zealand

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Abstract—The effects of nonsteroidal anti-inflammatory agents on superoxide production and granule enzyme release by human polymorphonuclear leukocytes stimulated with either formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe)) or immune complexes were investigated. Cytochrome *c* reduction and the release of lysozyme, β-glucuronidase, myeloperoxidase and gelatinase were measured. Auranofin, phenylbutazone, sulfasalazine and the phospholipase A_2 inhibitor, 4-bromophenacyl bromide, strongly inhibited these responses in fMet-Leu-Phe stimulated cells, at concentrations below 50 μM. Indomethacin, piroxicam, mefenamic acid, primaquine and quinacrine at 50–250 μM were inhibitory. Up to 1 mM ibuprofen and chloroquine inhibited superoxide production but had little effect on degranulation. With cells stimulated by IgG aggregates (immune complexes), up to 1 mM ibuprofen, mefenamic acid and piroxicam did not inhibit either response. Indomethacin, phenylbutazone, sulfasalazine and primaquine inhibited, but considerably higher concentrations were required than with fMet-Leu-Phe. Quinacrine inhibited superoxide production equally well with both stimuli but inhibited enzyme release only with fMet-Leu-Phe. Only auranofin, 4-bromophenacyl bromide, and the weakly effective chloroquine exerted approximately the same effect with both stimuli. D-Penicillamine did not affect enzyme release with either stimulus and interfered in the superoxide assay. Gelatinase release induced by fMet-Leu-Phe was affected to the same extent, or slightly more, than release of the other granule enzymes. With immune complexes, there was only modest inhibition of gelatinase release by any of the drugs at 250–1000 μM. Our results reinforce previous observations that many anti-inflammatory drugs affect neutrophil functions, but their effects vary with stimulus. The relative insensitivity of immune complex-induced responses to most of the drugs must be taken into account when considering their mode of action.

Polymorphonuclear leukocytes (PMN†) are an important part of the host defense system. They also contribute to tissue damage in chronic inflammation. In response to inflammatory stimuli, they release their granule enzymes and generate superoxide and other reactive oxygen metabolites, as well as synthesize inflammatory mediators such as leukotrienes.

A number of nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to modulate PMN responses. Effects on motility and aggregation [1–5], degranulation [3–12], superoxide production [3, 4, 10, 13–15], and leukotriene synthesis [16, 17] have been observed. A majority of the studies has been carried out with the chemotactic tripeptide formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) as stimulus, but it is already becoming clear that the effect of the drug can depend on the nature of the stimulus and, possibly, also on the source of the PMN [10, 13, 14, 18]. In chronic inflammatory diseases, immune complexes are an important PMN stimulant, either when free or associated with structural components such as cartilage or basement membrane [19–21]. Few studies have been carried out with NSAIDs and immune complex-stimulated PMN [6,11], and the one substantial one used rat PMN [18].

We, therefore, investigated how a broad range of anti-inflammatory drugs affect human neutrophils stimulated with immune complexes and compared their effects to those observed with fMet-Leu-Phe. We studied the two PMN responses most likely to contribute directly to inflammatory tissue damage: superoxide production and degranulation. β-Glucuronidase, myeloperoxidase and lysozyme were measured as markers for specific and azurophil granules. We also monitored the release of gelatinase, which is considered to be a marker for a third type of secretory granule, termed C-particles [22]. Selected drugs from different classes of NSAIDs were investigated: ibuprofen, indomethacin, piroxicam, mefenamic acid and phenylbutazone, the slow-acting anti-

* Address reprint requests to: Dr. C. C. Winterbourn, Pathology Department, Christchurch School of Medicine, Christchurch Hospital, Christchurch, New Zealand.

† Abbreviations: DMSO, dimethyl sulfoxide; Ig, immunoglobulin; NSAID, nonsteroidal anti-inflammatory drug; PBS, Phosphate-buffered saline; PMN, polymorphonuclear leukocytes; and SARD, slow-acting antirheumatic drug.

rheumatics (SARDs) auranofin, D-penicillamine and sulfasalazine, the antimalarials quinacrine, primaquine and chloroquine, and the phospholipase A_2 inhibitor 4-bromophenacyl bromide [10].

MATERIALS AND METHODS

Materials. Ficoll 400 and QAE-Sephadex A50 were purchased from Pharmacia, Uppsala, Sweden, and Hypaque from Sterling Pharmaceuticals, New Zealand. Human albumin was from Behring, F.R.G., and rabbit anti-human albumin antiserum was donated by Dr. J. G. Lewis (Christchurch Hospital, New Zealand). Immunoglobulin G (IgG) was purified from human serum by ammonium sulfate precipitation and ion exchange chromatography on QAE-Sephadex [23]. Auranofin was provided by Smith, Kline & French, N.S.W., Australia, and sulfasalazine by Pharmacia, N.S.W., Australia. Other chemicals were obtained from the Sigma Chemical Co., St. Louis, U.S.A.

Drug solutions were prepared daily: auranofin in ethanol; chloroquine, quinacrine and D-penicillamine in water; and the other drugs in dimethyl sulfoxide (DMSO). The ethanol or DMSO added (up to 0.2%) was shown not to alter cell viability, enzyme release, or superoxide production.

Preparation of PMN. Human PMN were prepared from the blood of normal donors by centrifugation through Ficoll-Hypaque, dextran sedimentation, and hypotonic lysis of contaminating red cells [24]. The cell suspensions contained 95–97% PMN and 3–5% eosinophils. Viability was greater than 98%, assessed by Trypan blue exclusion. Incubations were carried out in 10 mM phosphate-buffered saline (PBS), pH 7.4, supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mg/ml glucose.

Preparation of IgG aggregates and immune complexes. Aggregates of IgG were generated by heating purified IgG (25 mg/ml in PBS) at 63° for 20 min. Immune complexes were formed by adding 1 vol. of human albumin (5 mg/ml in PBS) to 3 vol. of rabbit anti-human albumin antiserum, and leaving at 20° overnight.

Granule enzyme release. PMN were suspended at 5×10^6/ml for stimulation with fMet-Leu-Phe, and at 10^7/ml with IgG aggregates. The suspensions were preincubated for 5 min at 37° with cytochalasin B (5 μg/ml; 0.1% DMSO) and with various concentrations of each drug. In the case of auranofin, preincubation was for 30 min, and cytochalasin B was added 5 min before the stimulus. Either fMet-Leu-Phe (10^{-7} M) or aggregated IgG (1 mg/ml, final concn) was added. After 10 min (fMet-Leu-Phe) or 15 min (IgG) at 37°, the cell suspensions were centrifuged at 1000 *g* for 5 min. From each supernatant fraction, 200 μl was assayed for lysozyme [25], 85 μl for β-glucuronidase [26], 25 μl for myeloperoxidase [26], 100 μl for lactate dehydrogenase [27], and 25 μl for gelatinase. Gelatinase was assayed by measuring the degradation of heat-denatured collagen [28], prelabeled with [^{3}H]$NaBH_4$ [29].

To check for interference in the enzyme assays, each of the drugs (at the equivalent of the highest concentration tested) was added to the cell supernatant fraction following stimulation with fMet-Leu-Phe. Primaquine decreased lysozyme activity by 15%, but none of the other drugs had any effect. β-Glucuronidase was unaffected by all the drugs except sulfasalazine and quinacrine which gave color interference at >250 μM. Myeloperoxidase activity was decreased in the presence of indomethacin (30% inhibition by 500 μM) and piroxicam (20% inhibition by 500 μM) but was not affected significantly by the other drugs. Therefore, β-glucuronidase was the azurophil granule marker measured with indomethacin and piroxicam, and myeloperoxidase at the higher concentrations of sulfasalazine and quinacrine. Sulfasalazine added to PMN supernatant caused an approximately 30% decrease in measured gelatinase activity. All the other drugs, at the highest concentration used, caused less than 15% inhibition.

Superoxide production. PMN (5×10^6 in 1 ml) were preincubated for 5 min at 37° with various concentrations of each drug (except auranofin, which was preincubated for 30 min) and ferricytochrome *c* (100 μM). fMet-Leu-Phe (10^{-7} M) was added or immune complexes (125 μg albumin/ml), and A_{550} of the solution was monitored continuously at 37° [30]. Superoxide production was calculated from the maximum rate of change using ε_{550} (reduced-oxidized) = 21.1 mM^{-1}. With both stimuli, rates became maximal after approximately 0.5 min and remained constant for 2–3 min. Immune complexes gave more consistent cytochrome *c* reduction rates than IgG aggregates and were used, therefore, in this assay.

The drugs were checked for interference in the assay by measuring their effects on cytochrome *c* reduction by a xanthine oxidase superoxide generating system. Assays were carried out in the same buffer as above, with 100 μM cytochrome *c*, 150 μM hypoxanthine, approximately 0.01 units xanthine oxidase and 400 units catalase per ml, and appropriate concentrations of each drug. In this system, sulfasalazine at >50 μM, and 4-bromophenacyl bromide at >2 μM, strongly inhibited cytochrome *c* reduction. However, the extent of inhibition did not depend on cytochrome *c* concentration, and both drugs also inhibited uric acid production by the xanthine oxidase system (monitored at 295 nm in the absence of cytochrome *c*), to approximately the same extent as cytochrome *c* reduction. It is concluded, therefore, that these drugs affect the xanthine oxidase and not the superoxide detection system. Some of the other drugs gave slight inhibition (<20%) of cytochrome *c* reduction, when used at the highest concentration to which the PMN were exposed. This was also largely accountable for in terms of inhibition of urate production. Anyway, these small decreases were much less than any attributable to the effects of the drugs on PMN stimulation.

RESULTS

The effects of the twelve drugs on neutrophil degranulation and on superoxide production are shown in Fig. 1. None, at the highest concentration tested, increased lactate dehydrogenase release above control values, indicating that non-specific cell damage did not occur. Results for only one azurophil marker (myeloperoxidase or β-glucuronidase) are

shown for each drug but, in cases where both were measured, the two curves were indistinguishable. Each drug inhibited release of lysozyme (present in azurophil and specific granules) and the azurophil marker by approximately the same extent, suggesting that there was no selectivity for one type of granule.

fMet-Leu-Phe stimulation. All the drugs normally classified as NSAIDs had some effect on fMet-Leu-Phe-stimulated PMN. Ibuprofen (Fig. 1a) gave only modest inhibition of superoxide production, and had very little effect on enzyme release, but the others all inhibited in the 10–250 μM range. Both responses were inhibited to approximately the same extent by either indomethacin (Fig. 1b) or mefenamic acid (Fig. 1c), but phenylbutazone (Fig. 1d) and piroxicam (Fig. 1e) each had a greater effect on superoxide production.

Of the SARDs, auranofin completely blocked superoxide production at 2.5 μM and strongly inhibited enzyme release at a slightly higher concentration (Fig. 1f). Sulfasalazine also stongly inhibited both responses (Fig. 1g). D-Penicillamine up to 500 μM slightly stimulated degranulation (Fig. 1h). Its effect on superoxide production could not be measured because it reacts directly with cytochrome *c*. Primaquine was the most inhibitory antimalarial, affecting superoxide production more than enzyme release (Fig. 1i). Quinacrine (Fig. 1j) had less of an effect on superoxide production, and chloroquine (Fig. 1k) gave no significant inhibition of enzyme release and only moderate inhibition of superoxide production. 4-Bromophenacyl bromide at less than 10 μM strongly inhibited both responses (Fig. 1l).

Immune complex stimulation. When immune stimuli were used, only auranofin (Fig. 1f) and 4-bromophenacyl bromide (Fig. 1l) and the weakly active chloroquine (Fig. 1k) inhibited as well as they did with fMet-Leu-Phe. D-Penicillamine (Fig. 1h) had no effect on enzyme release with either stimulus. Ibuprofen (Fig. 1a), mefenamic acid (Fig. 1c) and piroxicam (Fig. 1e), all of which gave some inhibition with fMet-Leu-Phe, had no effect or slightly enhanced degranulation and superoxide production with immune complexes. The other drugs inhibited the responses to immune complexes, but approximately five times more indomethacin (Fig. 1b), ten times more sulfasalazine (Fig. 1g), and ten or four times more phenylbutazone, to inhibit superoxide production and degranulation, respectively (Fig. 1d), were required than with fMet-Leu-Phe. Primaquine required a ten times higher concentration to inhibit superoxide production with immune complexes but gave the same inhibition of enzyme release with both stimuli (Fig. 1i). Quinacrine affected superoxide production to the same extent with both stimuli (Fig. 1j), but only inhibited enzyme release with fMet-Leu-Phe.

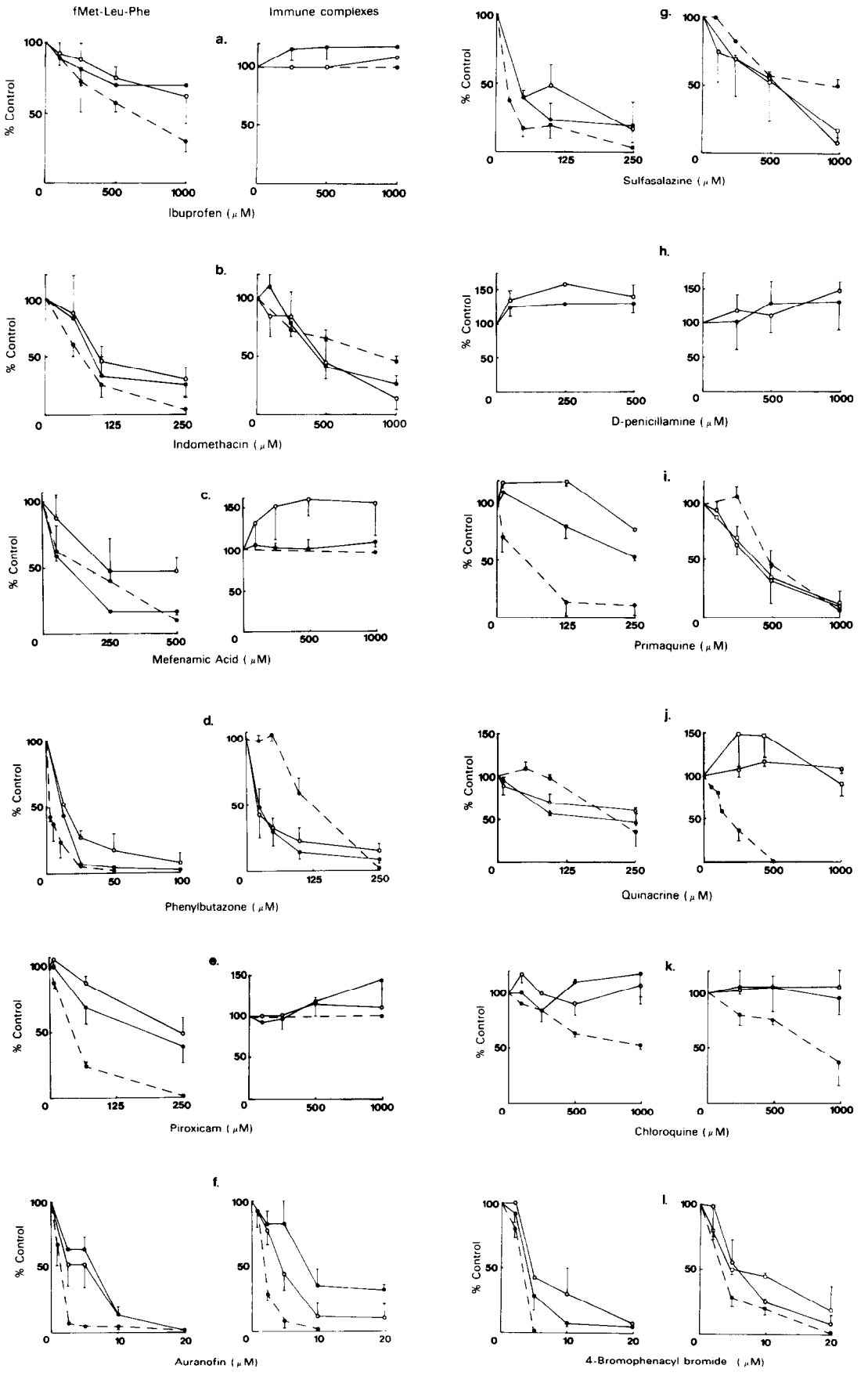
A possible reason why higher drug concentrations were required to inhibit responses to immune stimuli is that binding of the drug to the immunoglobulin reduced its effective concentration. To test whether this was the case with indomethacin, PMN were stimulated by fMet-Leu-Phe in the presence of 1 mg/ml native IgG. (The IgG solution had been ultracentrifuged to remove aggregates and was shown to be nonstimulatory.) At 100 μM, indomethacin decreased β-glucuronidase release by 57% compared

Table 1. Effects of anti-inflammatory drugs on PMN gelatinase release

Drug	Concentration (μM)	Gelatinase release (% control)	
		fMet-Leu-Phe	IgG aggregates
Indomethacin	250	47 ± 20	ND*
	1000	ND	80 ± 12
Ibuprofen	1000	ND	115 ± 4
Mefenamic acid	1000	ND	135 ± 32
Piroxicam	50	30 ± 18	ND
	250	3 ± 5	ND
	1000	ND	60 ± 9
Sulfasalazine	50	33 ± 8	ND
	250	8 ± 10	ND
	1000	ND	70 ± 19
Phenylbutazone	100	33 ± 27	ND
	250	ND	63 ± 3
D-Penicillamine	500	95 ± 13	ND
	1000	ND	56 ± 16
Auranofin	20	15 ± 3	67 ± 13
Chloroquine	1000	58 ± 18	69 ± 13
Primaquine	250	93 ± 5	ND
	1000	ND	36 ± 17
Quinacrine	100	73 ± 9	ND
	250	11 ± 12	ND
	1000	ND	149 ± 52
4-Bromophenacyl bromide	5	6 ± 6	ND
	20	6 ± 4	74 ± 16

Gelatinase was measured as described in Materials and Methods and is expressed relative to control values obtained with the same PMN preparation with no drug added. Results are the means ± SD for three to five observations.

* Not determined.


fMet-Leu-Phe
Immune complexes
a.
% Control
Ibuprofen (μM)
b.
Indomethacin (μM)
c.
Mefenamic Acid (μM)
d.
Phenylbutazone (μM)
e.
Piroxicam (μM)
f.
Auranofin (μM)
g.
Sulfasalazine (μM)
h.
D-penicillamine (μM)
i.
Primaquine (μM)
j.
Quinacrine (μM)
k.
Chloroquine (μM)
l.
4-Bromophenacyl bromide (μM)

with 60% in the absence of IgG, and 250 μM indomethacin decreased release by 75% compared with 76%. The effects of indomethacin on fMet-Leu-Phe induced lysozyme release were also unaffected by the presence of native IgG. Thus, interaction between the drug and IgG cannot explain the differences between the two stimuli.

Gelatinase release. Drug effects on gelatinase release were tested at the maximum concentrations shown in Fig. 1, and at lower concentrations where there was strong inhibition (Table 1). With fMet-Leu-Phe, a majority of the drugs inhibited gelatinase release to approximately the same extent as they inhibited release of the other granule enzymes. Piroxicam and quinacrine, however, inhibited gelatinase release more effectively. Results were generally less reproducible with gelatinase than the other enzymes, possibly because of the ease of spontaneous C-particle degranulation [22]. Ibuprofen and mefenamic acid were, on some occasions, inhibitory and on others not, and have not been included in the table.

With IgG-stimulated PMN, none of the drugs were potent inhibitors of gelatinase release. Indomethacin, sulfasalazine, auranofin and phenylbutazone, which almost fully inhibited release of the other enzymes, all had little effect on gelatinase release. The small inhibition by sulfasalazine may have been due to a direct effect on the enzyme assay. On the other hand, piroxicam, D-penicillamine and chloroquine inhibited release of gelatinase but not of the other enzymes.

DISCUSSION

A number of studies have shown that anti-inflammatory drugs can modulate the responses of PMN to fMet-Leu-Phe [3–5, 10, 11, 13–15]. Some, such as auranofin and phenylbutazone [3, 13–15, 31], have been shown to be potent inhibitors of both superoxide production and degranulation, and others, such as ibuprofen [4, 15], are less effective. Our results are in general agreement with these studies. In addition, we have shown that sulfasalazine and mefenamic acid are good inhibitors of fMet-Leu-Phe induced granule enzyme release, as well as inhibitors of superoxide production [5, 15]. No studies of the effects of antimalarials with fMet-Leu-Phe have been reported, although inhibition of PMN responses to opsonized zymosan and phorbol myristate acetate has been described [32, 33]. We found that primaquine and quinacrine inhibited both responses to fMet-Leu-Phe, with superoxide production being more sensitive than degranulation. However, the actions of antimalarials are not uniform, since chloroquine had little effect. The lack of effect of D-penicillamine is in agreement with results with latex-stimulated cells [34].

Studies with other stimulants, such as phorbol myristate acetate, concanavalin A and opsonized zymosan, have shown that PMN responses to NSAIDs are stimulus dependent. Few studies have examined PMN responses to immune complexes, yet these are one of the major inflammatory stimuli. We found considerable differences between the responses of immune complex (or aggregated IgG) and fMet-Leu-Phe stimulated cells. The only reported drug effects for human PMN and immune complexes are with auranofin. Potent inhibition of PMN responses was observed [7, 12], in agreement with our findings. Auranofin, along with chloroquine (which was much less potent), was the only drug to be equally effective with both stimuli. With the others, no clear pattern could be established. Some drugs did not inhibit superoxide production or degranulation with immune complexes, whereas they did with fMet-Leu-Phe. The rest required much higher concentrations to affect immune complex-induced responses. To further complicate the situation, some drugs were much more effective at inhibiting superoxide production compared with degranulation (or vice versa) with one stimulus, but not with the other.

The differences in drug concentration requirements for the two stimuli could be explained, in part, by some of the drugs inhibiting the binding of fMet-Leu-Phe to its receptor. Phenylbutazone, indomethacin, piroxicam and sulfasalazine have been shown to inhibit binding [3, 5, 13, 15], and immune complex-stimulated cells were less responsive to these drugs. However, phenylbutazone and sulfasalazine became inhibitory at higher concentrations, and, with phenylbutazone and primaquine, differences between fMet-Leu-Phe and immune complexes were much greater for superoxide than enzyme release. Inhibition of stimulus binding, therefore, is not the complete explanation. The phospholipase A_2 inhibitor 4-bromophenacyl bromide inhibited equally well with both stimuli. Hence, if phospholipase A_2 inhibition were the mode of drug action (which is one possibility with indomethacin [35]), differences in dose response would not be expected. A possible explanation could be that

Fig. 1. Effects of anti-inflammatory drugs on degranulation and superoxide production by PMN: (a) ibuprofen; (b) indomethacin; (c) mefenamic acid; (d) phenylbutazone; (e) peroxicam; (f) auranofin; (g) sulfasalazine; (h) D-penicillamine; (i) primaquine; (j) quinacrine; (k) chloroquine; and (l) 4-bromophenacyl bromide. Left-hand panels are for PMN stimulated with fMet-Leu-Phe. Right-hand panels for stimulation with IgG aggregates (degranulation) or immune complexes (superoxide production). Incubation conditions and assay procedures are given in Materials and Methods. Each point represents the mean ± SD of two to seven observations. In the absence of drug, mean (maximum) rates of superoxide production with fMet-Leu-Phe and immune complexes were 2.33 ± 1.24 and 1.49 ± 0.60 nmol/min/10^6 cells respectively. Control values for enzyme release per 10^6 cells were, for fMet-Leu-Phe and IgG aggregates, respectively: lysozyme 0.040 ± 0.016 and 0.019 ± 0.009 absorbance units/min; myeloperoxidase 1.45 ± 0.74 and 0.21 ± 0.10 absorbance units/min; and β-glucuronidase 30.6 ± 10.8 and 8.8 ± 4.7 μg phenolphthalein released/18 hr. Key: (●---●) superoxide production; (○——○) lysozyme; (●——●) β-glucuronidase; and (□——□) myeloperoxidase.

immune complexes induce phagocytic responses, which are less sensitive to inhibition than non-phagocytic responses. However, this is unlikely since cytochalasin B renders neutrophils incapable of phagocytosing immune complexes. This was verified in our study by electron microscopy which showed relatively few complete phagosomes in the stimulated cells compared with cells not exposed to cytochalasin B. We have also observed similar drug effects on PMN responses to surface-bound IgG [37]. Previous studies have shown that the effects of NSAIDs on PMN are not due simply to their ability to inhibit prostaglandin, and sometimes leukotriene, synthesis [2, 4, 10]. The stimulus–response coupling of PMN is a complex, multi-step process, as yet poorly understood. Undoubtedly the different drugs do not all act at the same step, and some may have more than one site of action.

There have been no previous studies of drug effects on the release of gelatinase, which is considered to be a C-particle enzyme. These granules are thought to be under separate control from the azurophil or specific granules, and to be released more readily [22], possibly to aid movement of the cells through tissues to an inflammatory site. We found that, with fMet-Leu-Phe stimulated cells, most drugs inhibited the release of gelatinase and the other granule enzymes to a similar extent, although piroxicam and primaquine had a greater effect on gelatinase release. However, all the drugs were relatively ineffective in inhibiting gelatinase release induced by immune complexes. Even phenylbutazone, auranofin, sulfasalazine and indomethacin, at concentrations which almost fully inhibited release of the other granule enzymes, inhibited gelatinase release by only 20–40%. These differential effects are not readily explicable if gelatinase is located in the specific granules, as has been reported recently [36]. Rather, they are supportive evidence for separate localization of gelatinase. Gelatinase is one of the PMN enzymes released in response to immune stimuli which is capable of degrading connective tissue proteins. The high NSAID concentrations required for even modest inhibition imply that modulation of immune complex-induced gelatinase release is likely to be physiologically unimportant.

The extent to which modulation of PMN function contributes to the therapeutic properties of anti-inflammatory drugs is not yet known. Some contribution would be expected, in view of the major role PMN play in the inflammatory response. Since their effects vary with different stimuli, the ability of drugs to modulate PMN responses at an inflammatory site will depend on which inflammatory mediators have the most influence on the cells. Immune complexes are a major physiological stimulus and, in diseases such as arthritis and glomerulonephritis, they can be embedded in cartilage or basement membrane matrix [19, 20]. Under such conditions, they induce release of PMN oxidants and enzymes at the surface where they may be inaccessible to scavengers and inhibitors [21, 30]. This results in degradation of the underlying matrix constituents [21]. Modulation of the secretion processes by drugs, therefore, could have a major protective effect. Extrapolation from *in vitro* to *in vivo* conditions must be made cautiously, especially with respect to efficacious drug concentrations. However, whereas the concentrations of most of the drugs that affected responses to fMet-Leu-Phe are potentially achievable physiologically [4], this is probably not the case for immune complexes. Auranofin and phenylbutazone, which were effective at low or intermediate concentrations, must be strongly favored to modulate immune complex-induced processes *in vivo* but, with the other drugs, such action is less likely.

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