Receptor-directed Inhibition of Chemotactic Factor-induced Neutrophil Hyperactivity by Pyrazolon Derivatives

DEFINITION OF A CHEMOTACTIC PEPTIDE ANTAGONIST

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ABSTRACT The two pyrazolon derivatives, phenylbutazone and sulfinpyrazone, selectively inhibit chemotactic peptide-induced effects on neutrophils. As they antagonize the induction of acute neutropenia in vivo and of cellular hyperadhesiveness, lysosomal enzyme release, hexose monophosphate shunt activity, and superoxide production in vitro, these effects occur with a specificity not shared with other prostaglandin biosynthesis inhibitors. Inhibition by these drugs resembles the competitive type of antagonism and occurs at concentrations attainable in vivo under clinical conditions. The locomotory machinery, the direction-finding mechanisms, and the basic metabolic machinery of the cell are unaffected. These drugs interfere with specific binding of the formylpeptide to its receptor on neutrophils.

INTRODUCTION

Recent research on the polymorphonuclear leukocytes (PMN)[1] has concentrated predominantly on their role in the host defense against invading microorganisms, resulting in the elaboration of the mechanisms involved in the phagocytic and microbicidal potency of these cells. The functional basis of many rare defects of these cells has been worked out elegantly along these lines (1, 2). However, such studies have also made it evident that normal PMN possess an "overkill" capacity that may be detrimental to the host if an inflammatory response is misdirected or excessive, leading to self damage (3). We have recently shown (4) that the exposure of PMN to synthetic chemotactic peptides at concentrations higher than those required for chemotaxis induce a highly reactive, probably cytotoxic (5) cellular condition. This is characterized by immobilizing hyperadhesiveness and spreading, accompanied by degranulation with enzyme release and stimulation of the hexose monophosphate pathway (HMP) activity, the latter phenomenon also reflecting superoxide production (6). Having in mind that mitigation of PMN overstimulation might be a promising way to modulate inflammatory processes, we looked for pharmacologic principles that are antagonistic to chemotactic peptide-induced PMN alterations.

METHODS

PMN were prepared from normal human volunteers by means of Ficoll-Hypaque density separation (96–99% PMN, 1–4% eosinophils). After hypotonic lysis of erythrocytes, PMN were washed twice in Gey's solution (Gibco Diagnostics, Glasgow, Scotland) and, if not stated otherwise, suspended in 95–100% pure heat-inactivated autologous plasma (4). *N*-formyl-Met-Leu-Phe (fMLP) was obtained from Bachem AG, Bubendorf, Switzerland. Phenylbutazone (PB) and sulfinpyrazone (SP) were gifts from Ciba-Geigy Ltd., Basel, Switzerland, and indomethacin was from Merck Sharp and Dohme, Rahway, N. J.; aspirin, inulin, and pepstatin A was purchased from Sigma Chemical Co. (St. Louis, Mo.). fMLP, SP, PB, and aspirin were dissolved in a small volume of 0.15 N NaOH and diluted in phosphate-buffered saline, pH 7.4 (PBS); fMLP, SP, and aspirin solutions were adjusted to pH 7.4 with 0.15 N HCl while PB was added to plasma in alkali solution. Three parts of indomethacin with one part Na_2CO_3 (wt/wt), was dissolved in PBS followed by adjustment of the pH with 0.15 N HCl. Drugs did not change plasma pH and plasma dilution by drug addition was ≤1% (vol/vol). Drug solutions were prepared fresh and added to experimental cell suspen-

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[1] *Abbreviations used in this paper*: fMLP, *N*-formyl-Met-Leu-Phe; HMP, hexose monophosphate pathway; K_d, dissociation constant; PB, phenylbutazone; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes; SP, sulfinpyrazone.

sions without any delay. PMN adherence, glucose oxidation by the HMP, and chemotaxis in Boyden chambers were quantitated as previously described (4). For chemotaxis, results are given in cells per high power field and represent means±SD of triplicates; in each case, experiments were repeated twice showing similar results. For glucose oxidation by the HMP, the incubation procedure was slightly modified in so far as the generation of $^{14}CO_2$ from [1-^{14}C]glucose was determined with PMN incubated in petri dishes (identical to adherence assay) instead of keeping them in shaken tubes. To ensure an air-tight system, covers of the dishes containing the CO_2-trapping device were sealed with petroleum jelly. The influence of SP/PB on latex- or Candida-stimulated HMP activity (as a measure of the phagocytosis-induced "respiratory burst" was assessed in shaking tubes. Latex was used as previously (4) and Candida albicans were grown under established conditions (7), heat-killed, washed once in PBS, and opsonized with fresh serum by rotating in a capped sterile tube for 30 min at 37°C; they were washed once more in PBS and added to the PMN in heat-inactivated plasma (with and without the drug) at a ratio of 10:1. As an indicator for lysosomal enzyme release during adherence, net release of a sensitive marker for secondary granule constituents, the PMN-associated vitamin B_{12} binding protein (8) was measured in the supernates of the petri dishes by the relative absorption of ^{57}Co-B_{12} (9). Appropriate controls excluded direct effects of the drugs tested on the B_{12} binding assay. Similar results were obtained by measuring lysozyme release (4).

Superoxide generation was assayed as described (10) using a dual-wavelength (550 vs. 540 nm) spectrophotometer (Shimadzu Seisakusho, Ltd., model UV 300, Kyoto, Japan). fMLP-induced O_2^- production was measured with PMN suspended in Krebs-Ringer-phosphate buffer (pH 7.4) containing 0.5 mg/ml glucose and 0.5% highly purified human albumin (Behring Corp., Marburg, West Germany). PMN (5×10^6) were kept in a stirred thermostated cell compartment; the continuously monitored change in absorbance, which followed the addition of fMLP, was always completed within 2–3 min, and was 28±5 nM (mean±SD, $n = 12$) ferricytochrome c reduced/5×10^6 PMN for maximal stimulation.

The radioligand binding assay for f-Met-Leu-[^{3}H]Phe (New England Nuclear, Boston, Mass.; 56.9 Ci/mmol sp act) was performed as described (11), except that the PMN suspension medium was autologous heat-inactivated plasma.

For studies in animals, New Zealand white rabbits (2.5–3.5 kg) were used, with sterile cannulas inserted in an ear vein for infusion of agents and into an ear artery for blood sampling.

RESULTS

In the rabbit model, intravenous administration of the potent chemoattractant fMLP (12) induces a profound transient granulocytopenia by excessive PMN margination (4, 13). Premedication of animals with as little as 10 mg/kg SP completely abolished an agranulocytotic response of 5 min duration after a bolus of 2.5 nmol of fMLP (Fig. 1A). Although similar results were obtained with the structurally related PB, the need for intramuscular administration meant that the drug was not suitable for exact dose-response studies. Nevertheless, a single injection of 50 mg/kg i.m. PB 5–6 h before fMLP administration abolished an identical agranulocytotic response completely. In contrast, two classic prostaglandin biosynthesis inhibitors, indomethacin (in doses as high as 25 mg/kg; Fig. 1A) and aspirin (up to 150 mg/kg; not shown), had no effect on this biologic activity of fMLP. To obtain information on the nature of the inhibition, untreated, as well as animals pretreated with a constant amount of SP, were given varying doses of fMLP. As illustrated in Fig. 1B, a parallel shift to the right of the dose-response curve was observed, as is expected for a competitive antagonist. Using a mean measured plasma level of SP of 270±40 (SD) μg/ml, which was found in samples obtained 20 min after drug administration ($n = 3$; method according to ref. 14), and assuming no essential immediate extravasation or destruction of fMLP, a rough estimation of the dissociation constant for the antagonist (K_d) (15) around a value of 21 μM can be obtained.

Because the fMLP-induced granulocytopenia in the rabbit might be an in vivo expression of an increase in PMN adhesiveness occurring intravascularly (4), the influence of PB and SP on fMLP-induced granulocyte adherence was studied in vitro. In all our in vitro systems, we kept the cells in 95–100% pure autologous plasma to represent the physiologic situation as closely as possible. As depicted in Fig. 2A, both drugs shifted the dose-response curve for adhesion induction with a similar potency to the right. From these curves, estimates for the K_d values are 80 μM (25 μg/ml) for PB and 30 μM (14 μg/ml) for SP, respectively. In addition, close parallelism was observed for the inhibition of the adherence-induced lysosomal enzyme release that predominantly affects constituents of the secondary granules (Fig. 2B). It is unlikely that PB and SP act by interfering with the arachidonic acid metabolism of PMN, as two potent inhibitors of this system were without any effect on a submaximal response to 0.1 μM fMLP in the petri dish assay (no significant influence on adherence nor on the release of B_{12}-binding protein or lysozyme): this was the case for pretreatment of PMN (*a*) with aspirin up to 300 μg/ml (1.7 mM), a dose that blocks prostaglandin biosynthesis in platelets, endothelial cells, and fibroblasts (16), and (*b*) with indomethacin up to 250 μg/ml (0.7 mM), a concentration far higher than that required to prevent thromboxane production (17) or the inhibition of the phospholipase A_2 activity (18) in PMN. Because PMN suspended in the presence of hyperadhesion-inducing concentrations of fMLP show marked alterations in oxygen metabolism (4, 6), the influence of the two pyrazolon derivatives on the fMLP-induced HMP activation was also assessed on petri dishes. Fig. 3A shows that PB was again a competitive antagonist for such HMP stimulation (K_d 13 μM). Fig. 3B represents an experiment in which various doses of the antagonist PB were used whereas the concentration of the agonist was kept constant. Virtually identical results were obtained with SP, whereas aspirin and indomethacin at the above mentioned concentrations were again ineffective.

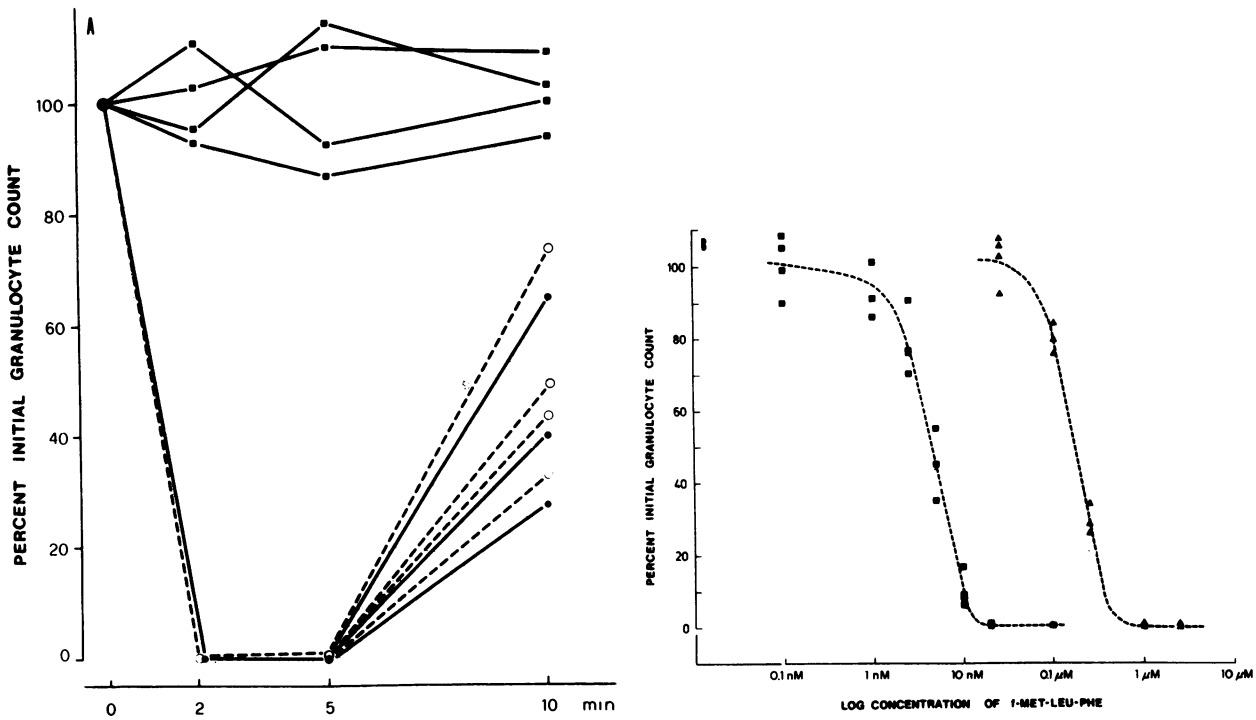

FIGURE 1 (A) Influence of indomethacin and SP on fMLP-induced granulocytopenia in rabbits. Agents were dissolved in 2.5 ml PBS and infused over 1.5 min. The PMN count found at 2, 5, and 10 min after a bolus of 2.5 nmol fMLP is expressed as percentage of the initial level. Curves represent single experiments in different animals. O - - - O, untreated animals; ● —— ●, animals pretreated with 25 mg/kg indomethacin; ■ —— ■, animals pretreated with 10 mg/kg sulfinpyrazone. Drugs were given 20 min before fMLP administration. (B) Inhibition of granulocytopenia-inducing capacity of fMLP in rabbits by SP. The mean of the PMN count found at 2 and 5 min after onset of fMLP infusion is expressed as percentage of the initial level (vertical axis) and plotted against the $\log_{10}$ of the estimated plasma molarity of fMLP (horizontal axis) assuming a rabbit plasma volume of 100 ml. Points represent single experiments in different animals. ■ - - - ■, untreated animals; ▲ - - - ▲, animals that had received 30 mg/kg SP 20 min before fMLP administration.

For technical reasons, fMLP-induced O_2^- production was assayed with PMN suspended in Krebs-Ringer phosphate buffer (containing glucose and albumin) instead of plasma. In this system, PB and SP were more potent (presumably due to lack of plasma binding of the drug). Thus, when cells were pretreated (20 min, 37°C) with 20 μg/ml SP, the parallel right shift of the dose-response curve reflected a K_d value of 7 μM for the antagonist. The same concentration of indomethacin (20 μg/ml) had no effect upon fMLP-stimulated O_2^- generation.

As compiled in Table I, PB and SP at concentrations used in these studies do not impede the basic locomotory activity (random migration) of PMN when tested in chemotactic chambers (A). However, they do blunt directed migration toward a chemotactic concentration of fMLP (B). In contrast, migration toward complement-derived chemotactic factors (10% inulin-activated plasma [4]) (C) was absolutely unimpeded. Furthermore, the drastically reduced PMN locomotion at adhesion-inducing concentrations of fMLP could be overcome, at least partly, in the presence of 250 μg/ml PB or SP; this was the case for random migration (D) as well as for chemotaxis toward 10% inulin-activated plasma (E).

In the light of previous studies that had shown inhibition of HMP activity by PB (19, 20), it was indicated to repeat such studies under our assay conditions using autologous heat-inactivated plasma instead of protein-free buffer solutions as the suspension medium. Applying two different stimuli for the activation of the HMP, one representing a complement-independent (latex), the other a complement (C3b)-dependent stimulus (opsonized Candida), we found absolutely no curtailment of [1-^{14}C]glucose oxydation in the presence of 250 μg/ml SP/PB. Latex-stimulated HMP activity amounted to 140±5 (SD) nM glucose/10^7 PMN per 30 min in the absence vs. 145±2 nM in the presence of SP/PB, and Candida-stimulated HMP activity was 208±12 nM in the absence vs.

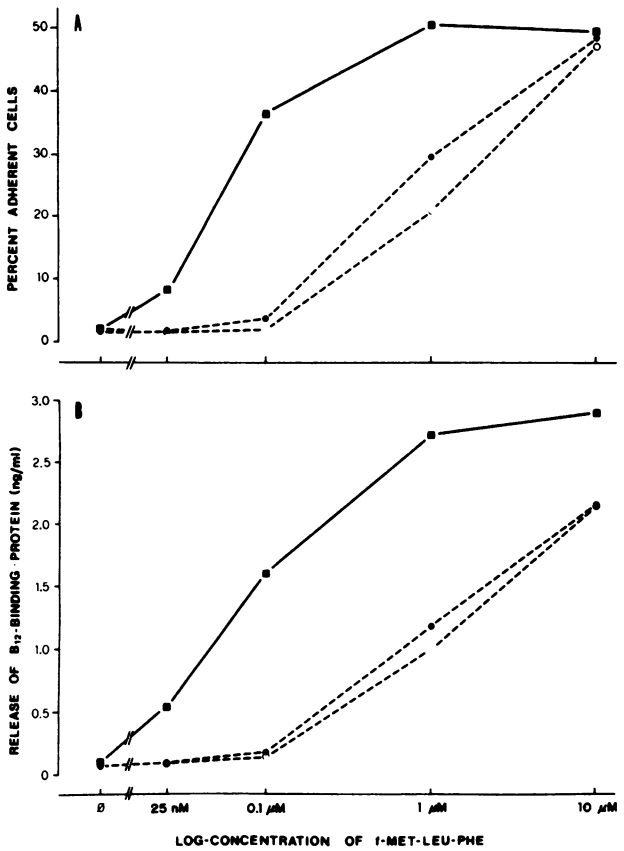

FIGURE 2 (A) Inhibition of fMLP-induced PMN adherence (■ —— ■) by 250 μg/ml (0.8 mM) PG (● - - - ●) or 250 μg/ml (0.62 mM) SP (○ - - - ○). Before addition of indicated concentrations of fMLP and plating on dishes, cells were preincubated for 20 min at 37°C with or without the drug. (B) As an indicator for the concomitant lysosomal enzyme liberation, the release of the secondary granule-associated vitamin B_{12} binding protein was measured in the supernates of the petri dishes (nanograms B_{12} bound per milliliter). Points represent mean of triplicates, with <5% variation between them; the experiments were repeated twice with PMN from different donors showing similar results.

212±24 nM in the presence of these drugs (resting HMP activity, which was also not inhibited by SP/PB, was 6±1 nM for these experiments; $n = 3$). Identical results, again with no inhibition of [1-^{14}C]glucose oxydation, were obtained in the presence of 30 μg/ml indomethacin. Therefore, as demonstrated above for the basic locomotory machinery, the basic metabolic machinery of the PMN seems not to be curtailed in the presence of the employed concentrations of SP/PB.

Because interference with PMN arachidonic acid metabolism did not seem to be the common denominator for the observed strong inhibitory potency of PB and SP on the chemotactic peptide-induced PMN hyperreactivity, we studied the influence of these drugs on the radioligand binding assay for f-Met-Leu-[^{3}H]-Phe. As can be seen from Fig. 4, PB and SP inhibit specific f-Met-Leu-[^{3}H]Phe binding to PMN. At 37°C, the concentration of PB and SP needed to provide a half-maximal inhibition of the binding (EC_{50}) amounted to 61 μg/ml (197 μM) and 29 μg/ml (72 μm), respectively. In contrast, aspirin (300 μg/ml) and indomethacin (20:100:200 μg/ml) resulted in no inhibition of specific radioligand binding (7±3 [SD]% and 6±2% inhibition, respectively). Since recent studies have made evident that, with respect to *N*-formyl peptides, human PMN show rapid internalization of the ligand-receptor complex (21), and, therefore, the measured alteration in cell-associated radioactivity could also be the consequence of an influence of the drugs on this or a later step in the uptake process, the binding and blocking studies for SP were also performed at 0°C, a temperature where internalization does not occur (21, 22). Under these conditions, with ligand-cell interaction for 60 min at 0°C, 10 times lower binding of f-Met-Leu-[^{3}H]Phe to the cells was observed causing a relative increase in nonspecific binding to 40–45%. Nevertheless, the curve for the SP-inhibited specific binding of the labeled peptide showed close parallelism to the one obtained at 37°C, and resulted in an EC_{50} of 40 μg/ml (98 μM).

From our results obtained in the experiments dealing with chemotaxis (Table I), it can be inferred that SP/PB may represent selective inhibitors for fMLP-induced PMN stimulation, and that these drugs do not impair responses to complement-derived stimuli. The following experiments were conducted to answer this question more clearly. First, in the rabbit model (23), rapid (1 min) administration of 2.5 ml i.v. of autologous inulin-activated plasma produced a complete agranulocytotic response of 5 min duration, with clearly visible PMN recirculation at 10 min. Pretreatment of animals with 50 mg/kg SP (procedure as given for Fig. 1 A, B) had absolutely no attenuating effect on this granulocytopenia ($n = 3$). Second, Fig. 5A–C depicts the in vitro results obtained with human PMN exposed to different concentrations of complement(inulin)-activated plasma in the presence and absence of SP (not shown are identical results obtained with PB). In contrast to the stimulation with fMLP (Figs. 2 and 3), no inhibitory potency can be ascribed to this drug with respect to complement-derived stimuli (presumably C5a). Third, because in our laboratory fMLP (1–10 nM) was a less potent chemotaxin (which means that it induced a smaller number of cells to migrate) than ≥10% inulin-activated plasma, the influence of PB/SP on PMN migration toward lower concentrations of activated plasma was also assessed. As visible in Table I (F and G) we found also no significant inhibition of such reduced migration at suboptimal concentrations of complement-activated plasma.

Further support for true selectivity of these two pyraz-

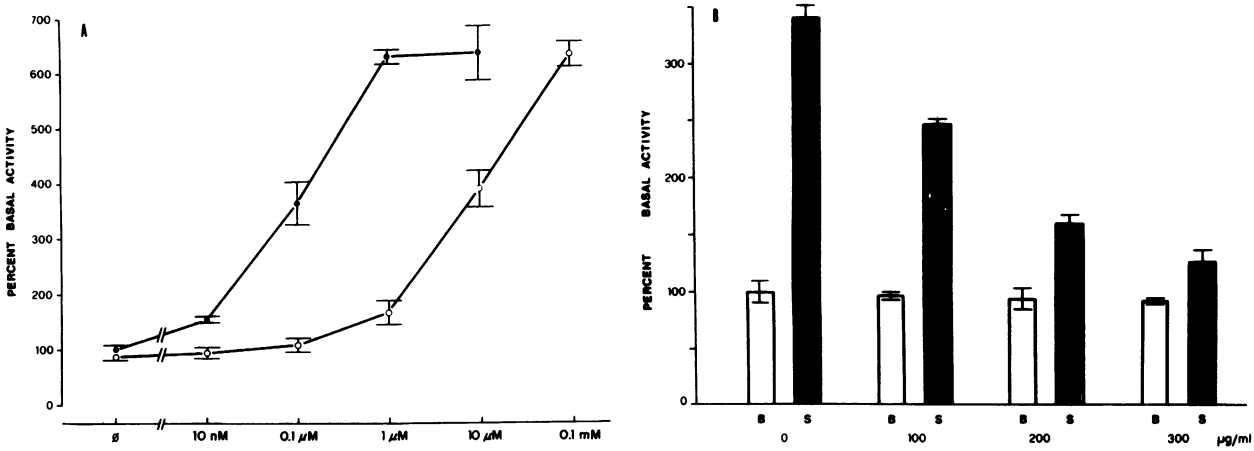

FIGURE 3 (A) Inhibition of fMLP-induced HMP activation (● —— ●) in the presence of 300 μg/ml (1 mM) PB (○ —— ○). PMN were preincubated (20 min, 37°C) in the presence or absence of the drug. Abscissa: $\log_{10}$ concentration of fMLP (Molars). Basal activity without fMLP and no drug added was 8.3±0.9 (SD) nM glucose/10^7 PMN per 30 min. Brackets denote mean±SD of triplicate determinations. (B) Inhibition of submaximal HMP activation by fMLP in the presence of various concentrations of PB. Conditions as given in A. **B**, basal activity in the absence of fMLP; no statistical difference without or with the indicated concentrations of PB. **S**, stimulated HMP activity in the presence of 0.1 μM fMLP; *P* between different concentrations of PB < 0.01 (Student's *t* test).

olon derivatives in their role as inhibitors of fMLP receptor-mediated PMN activation came from studies with pepstatin. Pepstatin, a naturally occurring microbial pentapeptide, is a potent chemotaxin for human PMN and shares a common receptor with fMLP on these cells (24). It does so despite its diverse primary structure and possession of an antigenicity different from the one elicited by fMLP (25). As compiled in Table II, pepstatin induced a comparable stimulation of PMN adhesiveness, enzyme release, and HMP activity, although these events occurred at a concentration roughly 100 times higher than that required for fMLP. Using this less effective agonist, quantitatively similar PMN responses were equally suppressed by SP/PB in a dose-dependent fashion.

DISCUSSION

Our results show that the two pyrazolon derivatives PB and SP possess, in vitro and in vivo, potent antagonis-

TABLE I
Influence of Phenylbutazone/Sulfinpyrazone on Random and Chemotactic Migration of Human PMN in Boyden Chambers

	Upper compartment	Lower compartment	Cells/HPF*	
			⊖	⊕
A	HIP	HIP	50±3	51±5
B	HIP	HIP + c-fMLP	131±10	63±12
C	HIP	HIP + 10% IAP	248±5	252±5
D	HIP + a-fMLP	HIP + a-fMLP	0.7±0.1	15±2
E	HIP + a-fMLP	HIP + a-fMLP + 10% IAP	10±0.5	54±6
F	HIP	HIP + 2.5% IAP	126±17	132±4
G	HIP	HIP + 1.25% IAP	100±4	89±8

* Abbreviations used in this table: HPF, high power field; HIP, autologous heat-inactivated plasma; c-fMLP, 1 nM f-Met-Leu-Phe; IAP, inulin-activated plasma added to HIP (vol/vol); a-fMLP, 200 nM f-Met-Leu-Phe.

⊖, in the absence of drug; ⊕, in the presence of 250 μg/ml SP or PB (SP and PB were used interchangeably giving results not different from each other). *P* between ⊖ and ⊕ > 0.05: A, C, F, G (Student's *t* test). *P* between ⊖ and ⊕ < 0.01: B, D, E.

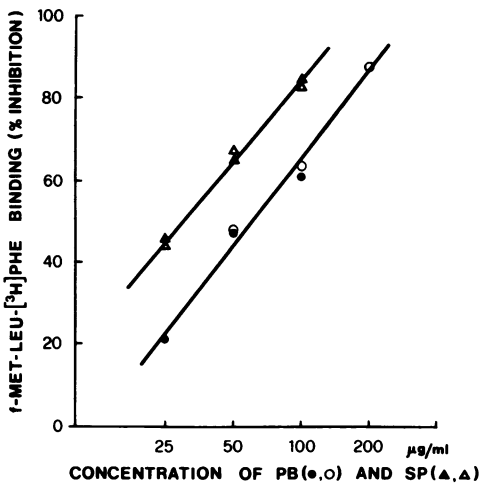

FIGURE 4 Inhibition of f-Met-Leu-[^{3}H]Phe binding to PMN by PB and SP. f-Met-Leu-[^{3}H]Phe at a concentration of 10 nM was incubated for 8 min at 37°C with human PMN which had been preexposed (15 min, 37°C) to PB (●, ○) or SP (▲, △) at the concentrations indicated on the abscissa (log scale). Results are expressed as percent inhibition of "specific" binding (11). "Nonspecific" binding, defined as ligand binding not inhibited by a 500-fold concentration of unlabeled fMLP, was always <5% of total bound counts. Each value represents the mean of triplicates; experiments were performed twice for each drug (open and closed symbols, respectively).

tic properties against chemotactic peptide-induced PMN alterations. The findings allow us (*a*) to define a clinically applicable pharmacologic antagonist of a receptor-specific PMN chemotoxin, and (*b*) to provide a novel mechanism of action for a nonsteroidal anti-inflammatory drug class that obviously interferes at an early step of the peptide-PMN interaction and is not dependent on the inhibition of the classic prostaglandin biosynthesis. SP and PB seem to be selective in competing with the agonists of a relatively well defined PMN receptor that shows specificity for *N*-blocked hydrophobic peptides (e.g., *N*-formyl peptides and pepstatin); obviously, these drugs do not interfere with identical biologic responses elicited by complement-derived chemotactic factors that are presumably mediated by the C5a receptor. In spite of receptor-directed specificity, the question whether SP and PB compete directly for peptide binding at the same receptor or if they modulate cell-peptide interaction by binding to an allosteric site cannot be answered by our study. Although we are well aware of the many discrepancies between presented and earlier results obtained in studies on pharmacological modulation of PMN function by nonsteroidal anti-inflammatory drugs, our findings stress the importance for adequate in vitro models. On the one hand, the strikingly close quantitative correspondence between the in vivo and in vitro inhibitory potency of SP and PB on fMLP-

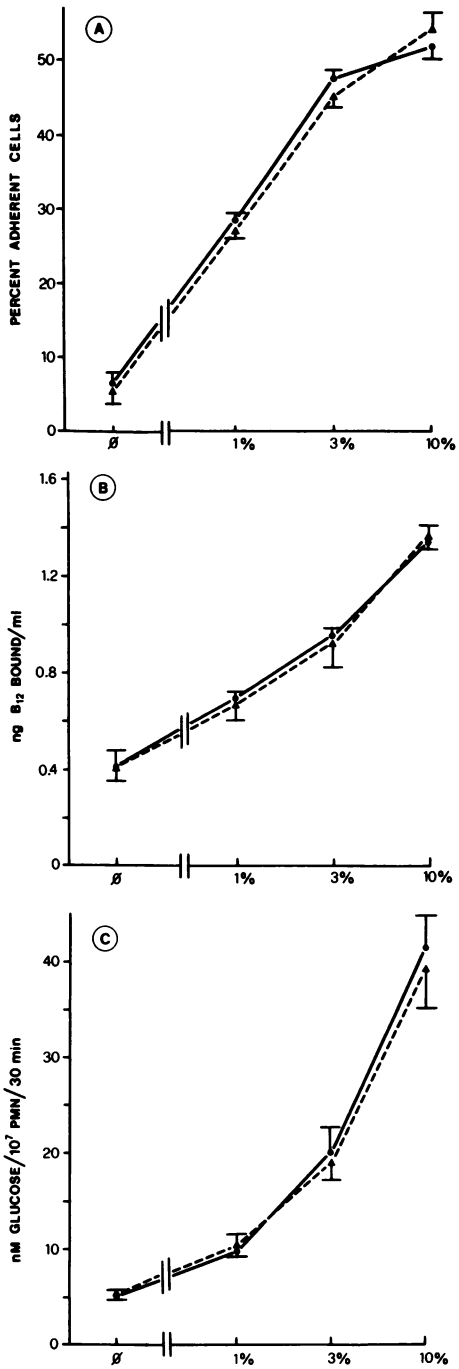

FIGURE 5 Complement-induced stimulation of PMN adherence (A), release of secondary granule constituents (B), and HMP activity (C) in the presence (● —— ●) and in the absence (△ - - - △) of SP. Before addition of the indicated concentrations of inulin-activated plasma (vol/vol; abscissa), cells were preincubated in autologous heat-inactivated plasma for 20 min at 37°C with or without 250 μg/ml of SP. Dilution of PMN suspensions resulting from addition of inulin-activated plasma was equalized by supplementing appropriate volumes of heat-inactivated plasma. Brackets denote mean ±SD of triplicate determinations.

TABLE II

Inhibition of Pepstatin-induced Stimulation of Neutrophils by Sulfinpyrazone/Phenylbutazone

Concentration of pepstatin	Adherence on petri dishes		Release of secondary granule constituents		HMP activity	
	% adherent cells		*pg B_{12} bound/ml*		*nM glucose/10^7 PMN per 30 min*	
	⊖	⊕	⊖	⊕	⊖	⊕
None	2.0±0.2	2.1±0.2	54±8.6	57±6.3	5.6±0.6	5.9±0.4
10 μM	17±1.8	2.4±0.5	481±37	166±17	34±3	7.4±1
100 μM	37±0.6	16±1.5	1,111±43	687±35	63±2	17±2

Data (mean±SD) are derived from three experiments performed in triplicates. ⊖, in the absence of drug; ⊕, in the presence of 250 μg/ml SP or PB (both drugs induced identical inhibition).
In the absence of pepstatin, no statistical difference exists between ⊖ and ⊕; in the presence of pepstatin, P of ⊕ vs. ⊖ is <0.001 (Student's t test).

induced neutropenia and adhesiveness, respectively, stands in favor of our methods. On the other hand, our results cast doubt on the physiologic relevance of studies showing inhibitory activity of indomethacin on several fMLP-induced PMN alterations in protein-free buffer systems (26–28), an activity that was completely lost upon addition of albumin (27, 28) or heat-inactivated serum (28).

Although it is certainly premature to decide firmly on the clinical relevance of our observations, especially as long as it remains unknown whether complement (C5a) or peptide receptor-mediated PMN hyperstimulation is more important for an uncontrolled and tissue damaging PMN reactivity, our findings provide us with a new and highly desirable (29) pharmacologic approach to anti-inflammatory therapy and may allow us to modulate the "fine tuning" of inflammation (30). We have indeed strong evidence from limited patient studies that pharmacological mitigation of hyperreactive PMN is achievable in vivo, which indicates that triggering of PMN by naturally arising undefined analogs of chemotactic peptides is also accessible to modulation by these drugs. In this context, preliminary in vitro results suggest that PMN adhesion to and spreading on immunoglobulin-coated surfaces is also decreased by PB/SP.

As recently demonstrated, SP is clinically effective in lowering the frequency of cardiac deaths after myocardial infarction (31). Because myocardial necrosis can be reduced by limiting the acute inflammatory response in the animal (32, 33) and since the reason for the beneficial action of SP in the postinfarction patient remains elusive and may be unrelated to its inhibitory effect on platelets (34), we might suggest that one possible way by which SP achieved its effect was by diminishing the PMN-dependent cytotoxic reactions.

Based on the fact that SP and PB probably act as competitive antagonists in our system, it is dictated by pharmacokinetic conditions that, in spite of similar in vitro potency of the two pyrazolon derivatives, PB remains the more potent drug in vivo. PB has a plasma half-life of 72 h, and presently used repeated clinical dosing (600 mg/d) leads to plasma levels between 50 and 150 μg/ml (35), whereas SP has a plasma half-life of 2.5 h, and a simple peroral dose of 200 mg leads to a maximal plasma level of ~20 μg/ml within 1–2 h (36). It is now a challenge to biochemical research to define the structure-effect principle more precisely and to look for chemical modifications of the indispensable structure in favor of higher effectiveness and lower toxicity.

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