Chemotactic Deactivation of Human Neutrophils: Protective Influence of Phenylbutazone

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The antiinflammatory drug, phenylbutazone (PBZ), has been studied in terms of its influence on chemotactic deactivation of human neutrophils. When PBZ was present during the time of preincubation of cells with N-formylmethionyl-phenylalanine (F-Met-Phe), loss of subsequent spontaneous mobility and chemotactic responsivity to F-Met-Phe did not occur. The action of PBZ to protect neutrophils from peptide-mediated chemotactic deactivation was found to involve in part its inhibitory influence on hexose monophosphate shunt activity and in part its antagonistic effect on interaction of peptide receptors with N-formyl peptide. Phenylbutazone interfered with binding of N-formyl-methionyl-leucyl-[\(^{14}\)H]phenylalanine to high-dose cytotoxyn, spontaneous migration and chemotactic responses to multiple attractants are depressed; exposure to lower doses of cytotoxyn results only in loss of chemotactic responsivity to the deactivating agent. We have attributed depression of the specific chemotactic response in the latter situation to loss of receptor function for the deactivating cytotoxyn (specific component) and losses of other migratory functions to events related to stimulation of the respiratory burst (nonspecific component).

To further explore the mechanisms of deactivation, we have assessed the influence of various antiinflammatory agents on this phenomenon. The present report describes the results of studies that demonstrate that phenylbutazone (PBZ) protects N-formyl peptide cytotoxyn-treated neutrophils against loss of spontaneous and chemotactic migratory functions and suggests a mechanism to account for this effect.

MATERIALS AND METHODS

Preparation of Cells

Blood was drawn from healthy human volunteers in heparinized syringes and neutrophils isolated by the method of Ferrante and Thong. The cell suspension medium used throughout this study was Hank's balanced salt solution (HBSS, GIBCO Laboratories, Grand Island, N.Y.).

Chemotactic Deactivation Studies

Spontaneous and chemotactic migratory responses were assessed by the migration under agarose method as previously described. The synthetic peptide cytotoxyn, N-Formyl-methionyl-phenylalanine (F-Met-Phe), was obtained from Andrus Research Corp., Bethesda, Md. Conditions for F-Met-Phe-mediated chemotactic deactivation were also as previously described. Phenylbutazone (4-butyI-1,2-diphenyl-3,5-pyrazinedione, PBZ) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co., St. Louis, Mo. Carbobenzoxy-phenylalanyl-methionine (CBZ-Phe-Met) was purchased from Vega Biochemicals, Tucson, Ariz. Phenylbutazone and CBZ-Phe-Met were dissolved in DMSO at a
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Studies of the Respiratory Burst

Superoxide-mediated reduction of cytochrome-C (Sigma) was measured spectrophotometrically as described by DeChatelet et al.\textsuperscript{20} Measurement of \textsuperscript{14}CO\textsubscript{2} production from \textsuperscript{14}C-glucose was done by standard methodology. In our assay, siliconized glass scintillation vials (Rochester Scientific Inc., Rochester, N.Y.) were prepared with 2 mCi of \textsuperscript{14}C-glucose of specific activity 5–10 mCi/\mu mole (New England Nuclear), and 10\textsuperscript{4} M F-Met-Phe or 1.3 mg serum opsonized zymosan in 0.5 ml total volume of phosphate-buffered saline (PBS) and prewarmed in a water bath to 37°C. One-half of these migratory functions observed in the absence of PBZ. Data are presented as mean ± standard error values.

RESULTS

PBZ-Mediated Protection Against Chemotactic Deactivation

The influence of PBZ on N-formyl peptide-induced loss of spontaneous and chemotactic migratory functions is illustrated by data from three experiments summarized in Fig. 1. Levels of loss of spontaneous and chemotactic migratory functions resulting from preexposure of neutrophils to 10\textsuperscript{4} M F-Met-Phe alone are illustrated by the horizontal shaded bars. Such treatment reduced subsequent spontaneous migration (SM) by an average 17% (range 14%–20%) and chemotaxis in response to F-Met-Phe (CT,FMP) by an average 33% (range 25%–39%). The presence of PBZ during the period of preexposure of F-Met-Phe produced a dose-dependent inhibition of these deactivation phenomena. At a concentration of 10\textsuperscript{4} MPBZ, essentially complete recovery of spontaneous mobility and chemotaxis in response to the dipeptide was observed. This protective influence of PBZ required that the drug be present during the period of exposure to F-Met-Phe, as addition of PBZ after exposure to peptide did not restore either migratory function (data not shown). Phenylbutazone was without cytotoxic effects as evidenced by its protective influence in these experiments. To further verify the drug’s nontoxic property and reversible nontoxic influence on migratory functions, we preexposed neutrophils to PBZ alone under comparable conditions, washed, and tested cell function in terms of spontaneous mobility (SM), chemotaxis in response to F-Met-Phe (CT,FMP), and chemotaxis in response to zymosan-activated serum (CT,ZAS). In one such experiment, mean and standard deviation values of triplicate tests of cells preincubated in control medium were 3.8 ± 0.3 cm for SM, 5.3 ± 0.1 cm for CT,FMP, and 4.5 ± 0.2
cm for CT,ZAS; values obtained for these functions following preincubation with 5 mM PBZ were 3.4 ± 0.2 cm, 5.3 ± 0.1 cm, and 4.5 ± 0.1 cm, respectively.

Direct Influence of PBZ on Migratory Functions

The observed ability of PBZ to protect neutrophils from desensitization to F-Met-Phe suggested that the drug may interfere with the interaction of F-Met-Phe with the neutrophil. To assess this possibility indirectly and to extend this consideration to include interaction of neutrophils with complement-derived attractant (CS5aC5bArg as zymosan-activated serum, ZAS), we tested the influence of PBZ incorporated into the agarose gel and cell suspension medium on neutrophil spontaneous mobility and chemotaxis in response to dipeptide and ZAS. The results of three such experiments are summarized by data in Fig. 2A. PBZ at concentrations of 10 and 100 μM reduced the projected distance of spontaneous migration from an average 3.2 cm in the absence of drug to 2.6 and 2.2 cm, respectively. In order to eliminate the contribution of loss of spontaneous mobility to effects of the drug on chemotactic responses, the influence of PBZ on chemotaxis toward these attractants has been compared by consideration of chemotactic differential (CT-SM) values. On this basis, data presented illustrate that the chemotactic differential obtained with F-Met-Phe as an attractant was essentially negated in the presence of 100 μM PBZ. In contrast, the drug had no such influence on these values with ZAS as the attractant. Data from one experiment are also provided in Fig. 2B, which demonstrate the influence of carbobenzyloxy-Phe-Met (CBZ-Phe-Met) in this

![Fig. 2.](image)

Fig. 2. (A) Dose effect of phenylbutazone (PBZ) incorporated into the agarose gel on human neutrophil spontaneous migration and chemotaxis in response to F-Met-Phe (FMP) and zymosan-activated serum (ZAS). Chemotaxis data are expressed as the chemotactic differential and chemotactic index, derived as noted. (B) Dose effect of carbobenzyloxy-phenylalanyl-methionine (CBZ-Phe-Met) on human neutrophil migratory responses. Data are presented as mean ± standard error values.

![Fig. 3.](image)

Fig. 3. (A) Influence of phenylbutazone (PBZ) on specific binding of F-Met peptide to human neutrophils. Neutrophils were incubated with 6 x 10⁻⁴ M F-Met-Leu⁻⁴³Phe at 0°C for 60 min in the absence or simultaneous presence of PBZ at the concentrations shown. Dose effects of other ligands for the peptide receptor, including F-Met-Leu-Phe (FMLP) and carbobenzyloxy-Phe-Met (CBZ-Phe-Met), are included for comparison. Data are presented as mean ± standard error values. (B) Influence of phenylbutazone (PBZ) on fate of F-Met peptide bound to human neutrophils. Neutrophils were preincubated with F-Met-Leu⁻³Phe at 0°C for a period of 60 min. Phenylbutazone, or an equal volume of PBZ-free medium, was then added and incubation was continued at 0°C. Total label remaining associated with sedimented cells was then determined at three subsequent time periods. Data are presented as mean ± standard error values.
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experimental protocol. CBZ-Phe-Met was chosen for this comparison because it is known to be a competitive antagonist for the N-formyl peptides. CBZ-Phe-Met similarly reduced the chemotactic differential value when F-Met-Phe was the attractant, but was without effect by this criterion when ZAS was the attractant.

Influence of PBZ on Binding of Radiolabeled Peptide and C5a

Results of the experiments described would suggest that PBZ may interfere selectively with the interaction of neutrophils with peptide ligand. To assess this possibility more directly, we have determined the influence of PBZ on binding of F-Met-Leu-[3H]Phe to human neutrophils. Extrapolating from the data presented in Fig. 3A, we calculated that PBZ at a concentration of approximately $4 \times 10^{-6}$ M will inhibit 50% of specific binding of F-Met-Leu-[3H]Phe at 0°C when drug and tripeptide are added simultaneously. For comparison, concentrations of F-Met-Phe, CBZ-Phe-Met, and F-Met-Leu-Phe required to achieve this result were approximately $2.8 \times 10^{-6}$ M, $5 \times 10^{-6}$ M, and $1.8 \times 10^{-8}$ M, respectively. The results of an experiment to assess the ability of PBZ to displace labeled tripeptide bound to the neutrophils in the absence of drug are illustrated by data in Fig. 3B. Forty-five minutes following addition of $10^{-8}$ M PBZ, total cell-associated label was reduced from an average 17,800 cpm to 5800 cpm.

Table 1. Influence of Phenylbutazone (PBZ) on Human Neutrophil Peptide Receptor Function

<table>
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<tr>
<th>Experiment Number</th>
<th>Presence PBZ</th>
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<tr>
<td></td>
<td>+</td>
<td>42,862</td>
<td>$8.5 \times 10^{-8}$ M</td>
</tr>
</tbody>
</table>

Means ± SEM.

Results summarized in Fig. 4 illustrate the influence of PBZ on binding of [125I]C5a. Neither PBZ nor F-Met-Leu-Phe was observed to interfere with binding of the complement-derived ligand to neutrophils. For comparison, the dose-related inhibitory influence of unlabeled C5a in this assay is also shown.

Mechanism of Inhibition of Peptide Binding by PBZ

To determine whether PBZ alters peptide receptor number or receptor affinity for peptide ligands, this inhibition phenomenon has been considered in terms of the Scatchard plot. Data from three such experiments are presented and summarized in Table 1. Receptor number in the absence of PBZ averaged $41,528 \pm 3538$ (mean ± SEM) per cell. In the presence of $10^{-8}$ M PBZ, the number of receptors averaged $42,862 \pm 2519$/cell, a statistically insignificant change ($p > 0.05$). Binding of F-Met-Leu-[3H]Phe in the absence of PBZ, expressed in terms of the dissociation constant ($K_0$), averaged $1.7 \times 10^{-8} \pm 0.3 \times 10^{-8}$ M (mean ± SEM). In the presence of PBZ, the $K_0$ value increased fivefold to $8.5 \times 10^{-8} \pm 1.1 \times 10^{-8}$ M ($p < 0.02$).

Mechanism of Protective Role of PBZ in Deactivation

The ability of PBZ to increase the dissociation constant of F-Met-Leu-Phe with the peptide receptor provides a clue to the mechanism by which PBZ prevents peptide-mediated chemotactic deactivation. Competitive antagonism of N-formyl peptide binding would reduce the amount of ligand bound to reduce the level of stimulation of the cells and also limit the down regulation of peptide receptors. Accordingly, we determined the influence of PBZ on F-Met-Phe-mediated stimulation of the respiratory burst measured as superoxide-dependent reduction of
cytochrome-C and as generation of $^{14}\text{CO}_2$ from $^{14}\text{C}\text{-}\text{l-glucose.}$ In the presence of $10^{-4} \text{ M PBZ}$ and $10^{-4} \text{ M}$ dipeptide, the respiratory burst by these assays was reduced by an average 98% and 65%, respectively ($p < 0.01$ for both determinations). Since PBZ may also more directly inhibit stimulation of this response through its ability to inhibit the hexose monophosphate shunt enzyme, glucose-6-phosphate dehydrogenase, the influence of PBZ on the respiratory burst stimulated by serum-opsonized zymosan was also tested. In two experiments, $10^{-4} \text{ M PBZ}$ inhibited phagocytosis-induced superoxide production and glucose metabolism by an average 14% ($p < 0.05$) and 10% ($p < 0.05$), respectively.

To assess the influence of PBZ on down regulation of peptide receptors we preincubated neutrophils with $10^{-4} \text{ M F-Met-Phe}$ in the absence and presence of $10^{-4} \text{ M PBZ}$ for 20 min at $37^\circ\text{C}$. washed the cells, and quantitated specific binding of F-Met-Leu-$[^{3}\text{H}]\text{Phe}$ at $0^\circ\text{C}$. Data obtained in two such experiments are presented independently in Fig. 5. The shaded bars represent the ability of neutrophils to specifically bind F-Met-Leu-$[^{3}\text{H}]\text{Phe}$ following each of the four pretreatment conditions. Statistical variability of these determinations is not shown, as each bar represents a value calculated by subtracting the average cpm representing nonspecifically bound tripeptide from the average cpm representing total bound ligand, with each mean being calculated from duplicate determinations. Standard error values of these means were consistently <5% of the respective mean values. We observed that preexposure of neutrophils to $10^{-4} \text{ M PBZ}$ had either no effect (experiment 1) or a marginal inhibitory effect (experiment 2) on subsequent specific binding of F-Met-Leu-$[^{3}\text{H}]\text{Phe}$. Preexposure to $10^{-4} \text{ M F-Met-Phe}$ alone reduced subsequent specific binding of the labeled tripeptide to levels representing 55% and 62% of the respective HBSS controls. Preexposure to $10^{-4} \text{ M F-Met-Phe}$ in the presence of $10^{-4} \text{ M PBZ}$ did not result in loss of subsequent specific binding of labeled tripeptide. The spontaneous and chemotactic migratory functions of the neutrophils following each

Fig. 5. Comparative influence of phenylbutazone (PBZ) on F-Met-Phe-mediated down regulation of peptide receptor function and chemotactic deactivation. Neutrophils were preincubated for 20 min at $37^\circ\text{C}$ in Hank's balanced salt solution, $10^{-4} \text{ M PBZ}$, $10^{-4} \text{ M F-Met-Phe}$, or PBZ and F-Met-Phe together. The cells were washed and tested for subsequent specific binding of F-Met-Leu-$[^{3}\text{H}]\text{Phe}$ (hatched bars) and spontaneous (SM) and chemotactic (CT, FMP) migratory responses (open bars). See text for description of binding data. Data representing the migratory functions are presented as mean projected distance of migration ± standard error values.
of these pretreatment conditions are represented by the open bars. In general, the pattern of loss of these migratory functions paralleled the pattern of loss of specific binding functions for F-Met-Leu-[3H]Phe in both experiments.

**DISCUSSION**

Data presented demonstrate that PBZ effectively protects the human neutrophil from F-Met-Phe-mediated chemotactic deactivation. Preincubation of neutrophils with PBZ together with F-Met-Phe prevented the loss of both spontaneous and chemotactic migratory functions that otherwise result from preexposure of neutrophils to the dipeptide (Fig. 1). The mechanism of this protective influence of PBZ appears to involve primarily inhibition of binding of F-Met-Phe to the neutrophil and secondarily inhibition of peptide-mediated stimulation of the respiratory burst.

The ability of PBZ to inhibit binding of N-formyl peptide to human neutrophils could occur, theoretically, by a number of alternative mechanisms. The drug might stimulate the neutrophil in some way to modify peptide receptor function or availability. Binding of PBZ to some membrane component adjacent to the receptor could affect receptor function. Further, PBZ might inhibit peptide receptor function by binding irreversibly with the peptide-binding site or another aspect (i.e., a PBZ-binding site) of the receptor to alter peptide-binding function. Finally, PBZ may function as a competitive antagonist of the interaction of N-formyl peptides with the peptide receptor.

Based on the experimental results described in this report, we believe that the ability of PBZ to inhibit binding of N-formyl peptide to human neutrophils reflects the drug's activity as a competitive antagonist. This possibility is strongly supported by multiple observations: (1) PBZ was able to eliminate the specific component of deactivation (Fig. 1); (2) PBZ, like CBZ-Phe-Met, inhibited a chemotactic response to F-Met-Phe but not ZAS (Fig. 2A,B); (3) PBZ inhibited specific binding of F-Met-Leu-[3H]Phe with a level of activity comparable to that of F-Met-Phe and CBZ-Phe-Met (Fig. 3A) and effectively displaced labeled tripeptide bound in the absence of PBZ (Fig. 3B); (4) PBZ-mediated inhibition of binding of labeled tripeptide was determined by Scatchard analysis to involve an increase in the $K_d$ for the ligand (Table 1), a pattern of inhibition of ligand binding characteristic of competitive antagonists; (5) PBZ did not affect binding of [125I]C5a (Fig. 4), demonstrating that the drug selectively influences peptide receptor function; and (6) preincubation of neutrophils with PBZ or PBZ and F-Met-Phe under conditions used for deactivation resulted neither in loss of chemotactic responsivity to F-Met-Phe nor in loss of ability of the treated cells to specifically bind F-Met-Leu-[3H]Phe (Fig. 5). These results and their interpretation complement those appearing in a recent report on this phenomenon by Dahinden and Fehr.

The structural aspects of PBZ (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) that would allow interaction of the drug with the peptide receptor as a competitive antagonist are not known. We have determined preliminarily that the structurally related compound, antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one), a pyrazol with a single phenyl group that also lacks the 4-butyl group, does not interfere with binding of F-Met-Leu-[3H]Phe to human neutrophils under comparable conditions. It is therefore possible that the aliphatic component of PBZ, together with the pyrazol ring and/or its phenyl groups could provide for such interaction. The interaction of PBZ with the peptide receptor cannot mimic that of N-formyl peptide, however, since PBZ was not active as a chemotactic agent.

The mechanism by which PBZ protects neutrophils from N-formyl peptide-mediated chemotactic deactivation most probably primarily reflects its ability to interfere with binding of the peptide ligand. Both the specific and nonspecific components of deactivation would be expected to be influenced by this activity. Inhibition of binding of N-formyl peptide would moderate the irreversible loss of peptide receptors that follows specific binding of peptide ligand. Data presented establish that the ability of neutrophils to specifically bind F-Met-Leu-[3H]Phe following preincubation with F-Met-Phe and PBZ is unaltered, whereas preexposure to dipeptide alone reduced specific binding of the tripeptide to approximately 60% of the control value (Fig. 5).

Inhibition of binding of peptide ligand would also moderate the level of stimulation of the respiratory burst to reduce loss of spontaneous mobility attributable in part to production of toxic products of oxygen metabolism. Complementing this mechanism of influence of PBZ must also be its more direct effect on the respiratory burst through inhibition of glucose-6-phosphate dehydrogenase activity. The greater inhibitory influence of PBZ on the superoxide production and hexose monophosphate shunt activity stimulated by F-Met-Phe (98% and 65%, respectively) than on these assays using opsonized zymosan as the stimulant (14% and 10%, respectively) would argue, however, this influence must play a secondary role in protection from loss of spontaneous mobility, since the effect of PBZ on this shunt enzyme should be independent of the stimulant.
The experiments described in this report provide an important example of drug-mediated modulation of a human neutrophil cytotaxin receptor function. Further, if PBZ, like pepstatin, binds directly to the peptide receptor, our concept of ligand specificity of this receptor must be modified to include compounds outside of the N-formyl peptide series. The clinical significance of these observations is not obvious, as the contribution of N-formyl peptide agonists to stimulation (and inhibition) of various neutrophil functions in vivo remains to be determined.

REFERENCES

Chemotactic deactivation of human neutrophils: protective influence of phenylbutazone

RD Nelson, JM Gracyk, VD Fiegel, MJ Herron and DE Chenoweth