

# Inhibition of Human Polymorphonuclear Leukocyte Function by 2-Cyclohexene-1-One

## A ROLE FOR GLUTATHIONE IN CELL ACTIVATION

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**ABSTRACT** 2-cyclohexene-1-one and diethyl maleate specifically decrease reduced glutathione (GSH) levels in human polymorphonuclear leukocytes (PMN) by direct conjugation, and by interaction with the glutathione-s-transferase system. Using these two nontoxic reagents we have examined the effect of decreased GSH levels on five parameters of PMN activation: superoxide generation, release of the lysosomal enzymes lysozyme and  $\beta$ -glucuronidase, and increases in the influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ . When PMN pretreated with 2-cyclohexene-1-one or diethyl maleate were incubated with formyl-methionyl-leucyl-phenylalanine (FMLP) or the proteolytic fragment of the fifth component of complement, C5a, agents that interact with surface membrane receptors, increases in all five parameters were inhibited in a dose-dependent manner. For  $\text{O}_2^-$  generation and lysosomal enzyme release the  $\text{ID}_{50}$  for 2-CHX-1 was 40–90  $\mu\text{M}$  corresponding with a 30–50% decrease in intracellular GSH. In contrast stimulation of treated PMN by the divalent cation ionophore A23187 or 5-hydroxyeicosatetraenoic acid was much less sensitive to depressed GSH; the  $\text{ID}_{50}$  for 2-cyclohexene-1-one was 1 mM or greater, corresponding with an 80–90% decrease in GSH. The effect of lowered GSH was not the result of decreased binding of FMLP to surface receptors because [ $^3\text{H}$ ]-FMLP binding studies demonstrated a two- to three-fold increase in the number of available binding sites. These data indicate that normal GSH levels are necessary for the transduction of the activation signal from the exterior to the interior of the PMN, but once initiated the activation sequence proceeds normally despite markedly lowered intracellular GSH.

## INTRODUCTION

Human polymorphonuclear leukocytes (PMN)<sup>1</sup> stimulated by chemotactic or phagocytic stimuli undergo a complex series of biochemical events that begin within seconds of the addition of the stimulus (1). Although many of these events have been studied in great detail, the exact interrelationships among the individual components of the response have not been elucidated.

A number of studies using relatively indirect methods have indicated that sulfhydryl groups (SH) in general and glutathione (GSH) in particular may be involved in the biochemical events that lead to PMN activation (2, 3). The interpretations of these studies, which involve the use of agents that bind to or oxidize free SH groups, may be criticized for the relative lack of specificity of the reagents used. For example, *N*-ethyl maleimide, an SH-alkylating agent, is capable of reacting with other functional groups, such as amino groups, under appropriate conditions (4). Diamide, which inactivates a number of PMN functions (2) is capable of oxidizing not only GSH, but also protein-bound SH groups (5). Other workers have examined the effects of exogenous GSH or other low molecular weight SH compounds on PMN function. Although these agents do have significant effects, particularly in situations where GSH levels have been lowered, the actual role these reagents play is not clear be-

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<sup>1</sup> Abbreviations used in this paper: 2-CHX-1, 2-cyclohexene-1-one; DEM, diethyl maleate; FMLP, formyl-methionyl-leucyl-phenylalanine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid;  $\text{O}_2^-$ , superoxide radical; PMN, polymorphonuclear leukocytes; SH, sulfhydryl groups.

cause many of these, most particularly GSH, do not readily enter the PMN.<sup>2</sup>

As part of an ongoing study of the role of GSH metabolism in human PMN function we have examined methods for specifically depleting GSH as a means of probing PMN function. We chose to use agents that interact with GSH by direct conjugation and via the glutathione-S-transferase system as a method of specifically decreasing intracellular GSH levels. These reagents are conjugated with glutathione by a thio-ether bond followed by cleavage of the  $\gamma$ -glutamyl and glycyl moieties of glutathione, *N*-acetylation of the cysteine, and excretion of the resulting mercapturic acid. This method affords the advantage that GSH levels can be reduced in a dose-dependent fashion with no increase in the levels of oxidized glutathione. Of several reagents capable of reacting with GSH enzymatically or nonenzymatically (6), 2-cyclohexene-1-one (2-CHX-1) (Fig. 1) and diethyl maleate (DEM) are the most potent nontoxic reactants in the PMN.

In the present study, we have examined the effects of 2-CHX-1 and DEM on a number of biochemical events associated with PMN superoxide generation and granule enzyme release. The data to be presented indicate a correlation between depletion of intracellular GSH levels and the functional responses induced by two chemotactic factors, formyl-methionyl-leucyl-phenylalanine (FMLP) and the proteolytic fragment of the fifth component of complement, C5a.

## METHODS

**Medium.** The medium used throughout this study was a Hanks' balanced salt solution supplemented with glucose, 1 mg/ml, and bovine serum albumin 1 mg/ml, pH 7.40. In those experiments utilizing 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), bovine serum albumin was eliminated from the media.

**Reagents and chemicals.** Ficoll, dextran, 5,5'-dithio-bis(2-nitrobenzoic acid), glutathione reductase from yeast, ferrocytochrome *c* (horse heart type III), FMLP, 3-phosphoglyceric-phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, adenosine 5'-triphosphate, 3-phosphoglyceric acid, and cyclohexanone were from Sigma Chemical Co. (St. Louis, Mo.). L-Cysteine-HCl was from Alfa Division, Ventron Corp. (Danvers, Mass.). Hypaque was from Winthrop Laboratories (New York). 2-Cyclohexene-1-ol and cyclohexene were from Aldrich Chemical Co. (Milwaukee, Wis.). A23187 was the generous gift of Dr. Richard Hamill, Eli Lilly Corp., Indianapolis, Ind. [<sup>3</sup>H]FMLP (56.9 Ci/mmol), [<sup>22</sup>Na]Cl (carrier-free), and [<sup>45</sup>Ca]Cl<sub>2</sub> (45 Ci/g calcium) were from New England Nuclear (Boston, Mass.). 2-CHX-1 and DEM were from ICN K & K Laboratories, Inc. (Plain View, N. Y.).

C5a was generated from human serum treated with zymosan particles in the presence of epsilon amino caproic acid as

previously described (7). 5-HETE was obtained biosynthetically from A23187-stimulated human PMN and purified by thin-layer chromatography and high-pressure liquid chromatography as previously described (8). FMLP was dissolved in dimethylsulfoxide (DMSO) at 1 mM and diluted further in medium; A23187 was dissolved in DMSO at 5 mg/ml and diluted further in medium. Cyclohexene was dissolved at 0.2 M in DMSO and diluted further in medium. At the concentrations used DMSO had no effect on either control or 2-CHX-1-treated PMN. Diethyl maleate, 2-CHX-1, 2-cyclohexene-1-ol, and cyclohexanone were dissolved directly in medium.

**PMN isolation.** PMN from normal volunteers were purified from heparinized peripheral venous blood by dextran sedimentation and Ficoll/Hypaque density gradient centrifugation as previously described (9). After two hypotonic lyses to remove contaminating erythrocytes the preparation contained 95–98% PMN with the remainder being small lymphocytes. The viability was >95% as judged by trypan blue dye exclusion. PMN were resuspended in medium and incubated for 15–60 min at 37°C to replenish GSH stores that may be depleted because of extensive washing in glucose-free media. The PMN were then exposed to appropriate concentrations of 2-CHX-1 or DEM and incubated for 30 min at 37°C. This time gave near maximum depletion of GSH levels.<sup>3</sup> The PMN were then washed two times in medium to remove residual drug and then used in GSH determinations or functional assays as described below.

**Glutathione assay.** PMN were sedimented at 400 g and resuspended at  $100 \times 10^6$ /ml in 0.1 M phosphate, 0.005 M EDTA, pH 7.4, and precipitated with 10% TCA containing 0.01 N HCl. After 30 min at 4°C, the precipitated proteins were removed by centrifugation at 2,200 g, and the supernate was extracted six times with 5 vol of H<sub>2</sub>O-saturated acidified diethyl ether. The resulting solution was assayed directly (total glutathione) or incubated with 0.02 M *N*-ethyl maleimide for 30 min at 23°C followed by removal of *N*-ethyl maleimide by ether extraction as described above. Total glutathione and oxidized glutathione (GSSG) were assayed by the method of Tietze (10) using GSH as a standard. GSH was calculated from the difference between total glutathione and GSSG. In control experiments >95% of GSH added to PMN before addition of TCA was recovered in the GSH assay.

**Estimation of SH reactivity of DEM and 2-CHX-1.** To demonstrate that 2-CHX-1 or DEM were specific for the SH group of glutathione, GSH, cysteine, human serum albumin, and alpha lactalbumin were reacted with 2-CHX-1 or DEM for 30 min at 37°C. Free SH groups were then detected using 5,5'-dithio-bis(2-nitrobenzoic acid) (11). A decrease in titratable SH groups was taken as a measure of 2-CHX-1 or DEM reactivity. The decrease in free SH groups because of disulfide formation was assayed in control experiments and was subtracted from the results presented.

Additional experiments were done using a nuclei-free sonicate of PMN ( $6 \times 10^5$ -bursts Biosonics III sonicator, Bronwill Scientific, Rochester, N. Y., followed by centrifugation at 120 g for 4 min) to test for glutathione-S-transferase activity. Cell sonicate alone or combined with GSH or cysteine was incubated with or without 2-CHX-1 or DEM for 30 min at 37°C. A decrease in free SH groups was determined as outlined above. The difference between the decrease in titratable SH groups in the presence or absence of PMN non-

<sup>2</sup> Fischman, C., J. McDonald, and H. J. Wedner. Unpublished observations.

<sup>3</sup> Wedner, H. J., and C. Fischman. Unpublished observation.

nuclear sonicate provided a measure of PMN glutathione-s-transferase activity.

To explore further the protein SH reactivity of 2-CHX-1 the effect of this reagent on the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a highly sensitive SH enzyme was examined. GAPDH activity was measured by the conversion of 3-phosphoglyceric acid to glyceraldehyde-3-phosphate in the presence of GAPDH, 3-phosphoglyceric phosphokinase, ATP, and reduced  $\beta$ -nicotinamide adenine dinucleotide. The reaction mixture contained in a final volume of 3.0 ml: 0.1 M triethanolamine buffer pH 7.6, 20  $\mu$ mol 3-phosphoglyceric acid, 10  $\mu$ mol L-cysteine, 5  $\mu$ mol  $\text{MgSO}_4$ , 0.35  $\mu$ mol  $\beta$ -NADH, 3.4  $\mu$ mol ATP, 5 U of 3-phosphoglyceric phosphokinase, and 0.03 U of GAPDH. The reaction was initiated by the addition of GAPDH, and the decrease in  $\beta$ -NADH was followed spectrophotometrically at 340 nm using a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). 2-CHX-1 was added to the reaction mixture before the addition of GAPDH or was incubated with GAPDH for 90 min at 4°C before the addition of GAPDH to the assay mixture. Results are expressed as moles  $\beta$ -NADH oxidized per minute.

**Superoxide generation.** Superoxide generation was assayed by the reduction of ferricytochrome *c* as previously described (12). Briefly, PMN ( $1.8\text{--}3.0 \times 10^6$ ) were incubated with ferricytochrome *c* (76  $\mu$ M) and the appropriate concentration of stimulator in a final volume of 0.6  $\text{cm}^3$  for 5–15 min at 37°C. The reaction was terminated by centrifugation for 30 s in a microcentrifuge (Beckman Instruments, Inc., Fullerton, Calif.); 0.2 ml of the supernate was diluted to 3.0 ml with 0.1 M potassium phosphate buffer (pH 7.4) and reduced cytochrome *c* detected spectrophotometrically at 550 nm in a Gilford 250 spectrophotometer (Gilford Instrument Laboratories Inc.).

**$^{22}\text{Na}^+$  and  $^{45}\text{Ca}^{2+}$  uptake studies.** PMN ( $1.8 \times 10^7$ ), preincubated with 2-CHX-1, DEM, or media, were incubated with 1.4  $\mu\text{Ci/ml}$  [ $^{22}\text{Na}$ ]Cl or 1.4  $\mu\text{Ci/ml}$  [ $^{45}\text{Ca}$ ]Cl<sub>2</sub> in a total volume of 1.8 ml in the presence or absence of stimuli for 5 min at 37°C. Then, 0.5 ml of the PMN suspension was layered over 0.7 ml of Versilube F-50 oil (Harwick Chemical Corp., Akron, Ohio) in 1.5-ml plastic microcentrifuge tubes and centrifuged at 8,000 g for 60 s. Separation of cells from media occurred in <10 s. The cell pellet was excised and either counted in a gamma counter (Nuclear Chicago Corp., Des Plaines, Ill.) (for  $^{22}\text{Na}^+$  studies) or dissolved in 0.5 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) and counted in 10 ml of Aquasol-2 (New England Nuclear) in a Nuclear Chicago liquid scintillation counter (for  $^{45}\text{Ca}^{2+}$ ). To correct for the amount of isotope in the trapped extracellular fluid, a zero time set was included in each experiment and those counts per minute subtracted in calculating the results.

**Enzyme release studies.** Release of lysozyme,  $\beta$ -glucuronidase, and lactic dehydrogenase from 2-CHX-1-treated or control PMN was assayed as previously described (13).

**[ $^3\text{H}$ ]FMLP binding.** Binding of [ $^3\text{H}$ ]FMLP to 2-CHX-1 and DEM-treated or control PMN was performed as previously described (14). In this assay nonspecific binding defined as residual [ $^3\text{H}$ ]FMLP binding in the presence of a 250-fold excess of unlabeled FMLP was 15–20% of the total counts bound. Binding studies were carried out for 5 min.

**Calculations.** For each assay, the percent inhibition was calculated as follows:

Percent inhibition

$$= \left[ 1 - \left( \frac{\text{stimulus} - \text{control with drug}}{\text{stimulus} - \text{control without drug}} \right) \right] \times 100.$$

## RESULTS

**Intracellular glutathione levels.** PMN incubated with 2-CHX-1 at concentrations from 1  $\mu$ M to 1 mM for 30 min at 37°C showed a dose-dependent decrease in intracellular total GSH and reduced GSH levels (Fig. 2A). At 1 mM there was an 87% decrease in GSH. Similar results were seen for DEM (Fig. 2B) although this reagent is somewhat less potent on a molar basis. Despite this marked loss of intracellular glutathione, there was no decrease in cell viability for as long as 180 min as judged by trypan blue dye exclusion and lactic dehydrogenase release, and by the observation that little or no GSH leaked from the PMN during the course of the incubations (data not shown). In resting PMN, GSSG represented 1.3% of total GSH, and there was a concomitant fall in GSSG as GSH levels fell (Fig. 2B). The 2-CHX-1 analogues, 2-cyclohexene-1-ol, cyclohexanone, and cyclohexene, had no effect on PMN GSH levels (data not shown).

**Combination of 2-CHX-1 and DEM with free SH groups.** Several reports have indicated that 2-CHX-1 is specific for glutathione SH groups (15). To examine this question directly in the human PMN, GSH and cysteine alone or with PMN supernate were reacted with 2-CHX-1. As shown in Table I, 2-CHX-1 was able to combine with GSH in a nonenzymatic fashion. In contrast this reagent was unable to combine with cysteine or with cellular protein-bound SH groups. 2-CHX-1 was also unable to combine with the SH group of human serum albumin or  $\alpha$ -lactalbumin (not shown). When GSH and PMN nonnuclear sonicate were reacted with 2-CHX-1, there was a 2.3-fold increase in the amount of GSH conjugated, indicative of glutathione-s-transferase activity. The increase in GSH conjugation was completely destroyed by boiling the PMN nonnuclear sonicate for 30 min. Similar results were seen with DEM (not shown).

The ability of 2-CHX-1 to interact with protein SH groups was further examined using GAPDH, which is highly sensitive to SH-reactive reagents. As shown in Table II 2-CHX-1 had no effect on GAPDH activity either when added to the reaction mixture before the addition of GAPDH or when preincubated with GAPDH for 90 min at 4°C. This study also confirms the inability of 2-CHX-1 to react with cysteine be-

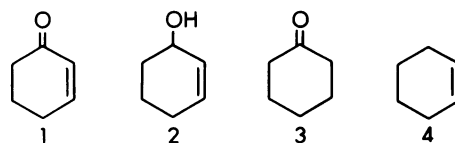


FIGURE 1 Structure of 2-cyclohexene-1-one and related compounds. (1) 2-cyclohexene-1-one. (2) 2-cyclohexene-1-ol. (3) Cyclohexanone. (4) Cyclohexene.

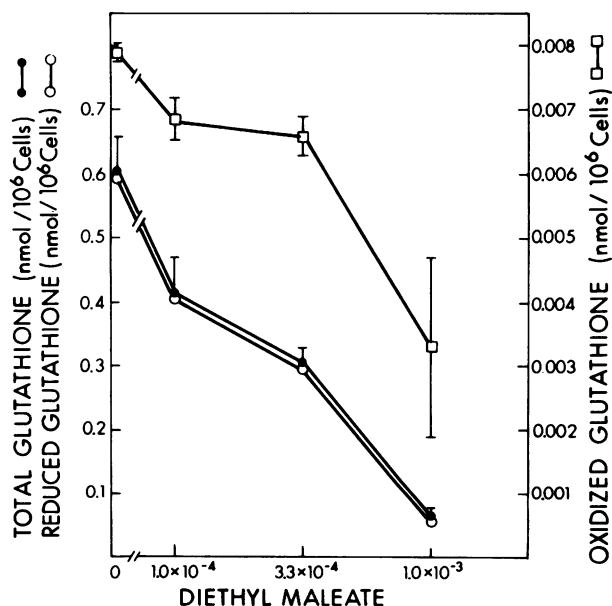
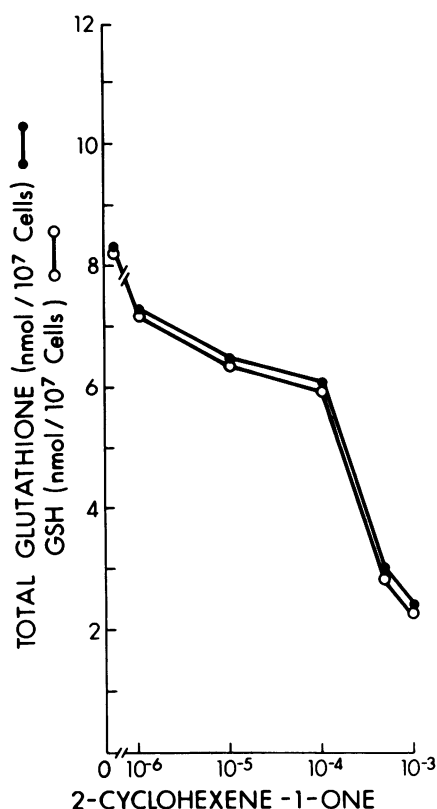


FIGURE 2 Effect of 2-cyclohexene-1-one and diethylmaleate on total and reduced glutathione in human PMN. Human PMN were incubated with varying concentrations of 2-CHX-1 or DEM for 30 min in complete media. After two washes in complete media the cells were suspended in 0.1 M phosphate, 0.005 M EDTA pH 7.4 and disrupted in 10% TCA. Total and reduced glutathione were assayed as described in the text. (A) Effect of 2-CHX-1. (B) Effect of DEM.

cause this reagent is necessary for GAPDH activity under the conditions tested.

**Superoxide generation and granule enzyme release.** The effect of treatment with 2-CHX-1 on  $O_2^-$  generation and granule enzyme release are shown in Figs. 3 and 4 and Table III. As displayed in Fig. 3, preincubation of the PMN with 2-CHX-1 (10  $\mu$ M to 1 mM) caused dose-dependent inhibition of  $O_2^-$  release induced by optimal doses of FMLP (0.1  $\mu$ M) and C5a (10  $\mu$ l/ml) (15). The doses of 2-CHX-1 causing 50% inhibition ( $ID_{50}$ ) of the FMLP- and C5a-stimulated responses were  $\sim$ 40  $\mu$ M and 50  $\mu$ M, respectively. In contrast, 2-CHX-1 pretreatment of the cells was much less effective in inhibiting  $O_2^-$  generation induced by A23187, 2  $\mu$ M ( $ID_{50}$  0.9 mM). The incubation periods (5 min for FMLP and C5a, 15 min for A23187) represent times by which  $O_2^-$  generation was essentially complete (16).

The inhibition of FMLP-induced  $O_2^-$  generation by 2-CHX-1 could not be overcome by the simultaneous preincubation of cells with 2-CHX-1 and either 2-mercaptoethanol (1 mM) or cysteamine (1 mM), agents that might be expected to maintain free SH groups (data not shown).

FMLP-stimulated  $O_2^-$  generation was unaffected by 6-diazo-5-oxo-L-norleucine (1 mM), an inhibitor of  $\gamma$ -glutamyl-transpeptidase activity (17), suggesting that the role of GSH in FMLP-induced  $O_2^-$  release was unrelated to transpeptidation.

DEM also decreased superoxide generation in response to FMLP (0.1  $\mu$ M) with an  $ID_{50}$  of 0.23 mM (Table III). As with 2-CHX-1 there was much less effect on superoxide generation induced by A23187; 50% inhibition was not achieved at any concentration of DEM tested.

TABLE I  
Combination of 2-CHX-1 with Free SH Groups

SH compound	PMN sonicate	SH Groups conjugated
nmoles		
Glutathione	-	8.3 $\pm$ 5.2
Glutathione	+	19.2 $\pm$ 2.0
Cysteine	-	0.02 $\pm$ 0.01
Cysteine	+	0.01 $\pm$ 0.01
—	+	0

Glutathione (0.1 mM) or cysteine (0.1 mM) was incubated with 0.1 mM 2-CHX-1 with or without 10  $\mu$ l of PMN nonnuclear sonicate. After 30 min at 37°C the number of free SH groups was quantitated using 5,5'-dithio-bis (2-nitrobenzoic acid) and compared with the number of free SH groups in the absence of 2-CHX-1. Results presented are the mean $\pm$ SEM of three experiments done in triplicate.

\* The low molecular weight SH compounds were omitted and the effect of 2-CHX-1 on cellular free SH groups examined. For this condition the PMN nonnuclear sonicate was dialyzed to remove GSH and cysteine before incubation.

TABLE II  
Effect of 2-CHX-1 on Glyceraldehyde-3-phosphate Activity

Enzyme preincubation	Inhibitor	Glyceraldehyde-3-phosphate dehydrogenase
		nmol NADPH oxidized/min
None	None	6.50±0.10*
None	2-CHX-1 10 $\mu$ M	6.75±0.45
None	2-CHX-1 0.1 mM	6.25±0.65
None	2-CHX-1 1 mM	6.55±0.15
Buffer	None	5.35±0.25
2-CHX-1 0.1 mM	1.67 $\mu$ M†	5.30±0.30

Glyceraldehyde-3-phosphate dehydrogenase activity, assayed as described in the text, was examined in the presence of the indicated concentrations of 2-CHX-1. Purified glyceraldehyde-3-phosphate dehydrogenase was utilized directly or after a 90-min preincubation with 0.1 mM 2-CHX-1 or 0.1 M triethanolamine buffer pH 7.6.

\* Mean±SD.

† Represents residual 2-CHX-1 from the enzyme preincubation.

As shown in Fig. 4, 2-CHX-1 also caused dose-dependent inhibition of FMLP (0.1  $\mu$ M), and C5a (10  $\mu$ l/ml) induced lysozyme release in the presence of 5  $\mu$ g/ml  $\beta$ -cytochalasin. The  $ID_{50}$  for 2-CHX-1 were 70  $\mu$ M and 60  $\mu$ M for the FMLP and C5a-stimulated responses, similar to the drug's  $ID_{50}$  for  $O_2^-$  generation. Pretreatment of the cells with 2-CHX-1 was a substantially less potent inhibitor of A23187 (2  $\mu$ M)-induced enzyme release with an  $ID_{50}$  of >1 mM. A similar effect of 2-CHX-1 on the stimulated release of  $\beta$ -glucuronidase activity was observed (Fig. 4B). The release of lactic dehydrogenase, a cytoplasmic enzyme, was not increased above control levels under any of the conditions outlined.

The effects of three analogues of 2-CHX-1, 2-cyclohexene-1-ol, cyclohexanone, and cyclohexene (shown in Fig. 1), were also tested. None of the analogues altered PMN GSH levels or had any effect on FMLP-stimulated  $O_2^-$  generation or granule enzyme release (data not shown).

**[ $^{22}Na^+$ ] and [ $^{45}Ca^{2+}$ ] uptake.** Activation of human PMN by FMLP, C5a, and A23187 has been shown to be associated with a transient increase in [ $^{22}Na^+$ ] and [ $^{45}Ca^{2+}$ ] uptake by the cells, the kinetics of which are similar to those of  $O_2^-$  generation and granule enzyme release (12, 13). As shown in Fig. 5, 2-CHX-1 pretreatment inhibited FMLP (0.1  $\mu$ M)-induced [ $^{22}Na^+$ ] uptake with an  $ID_{50}$  of 0.3 mM. In contrast, 2-CHX-1 had much less effect on [ $^{22}Na^+$ ] uptake induced by 20  $\mu$ M A23187 at 5 min with only 24.6% inhibition noted with 2-CHX-1 (1 mM), the highest dose tested. Similar effects of 2-CHX-1 were observed when suboptimal

concentrations of the stimuli were employed: FMLP (10 nM) and A23187 (2  $\mu$ M).

As shown in Table IV, incubation of the cells with 2-CHX-1 inhibited FMLP- and A23187-induced [ $^{45}Ca^{2+}$ ] uptake at 5 min comparable with those effects observed on stimulated [ $^{22}Na^+$ ] uptake (Fig. 5).

**[ $^3H$ ]FMLP binding studies.** Fig. 6 displays the results of experiments to test the effect of 2-CHX-1 on [ $^3H$ ]FMLP binding to PMN. Over the concentration range 33  $\mu$ M to 1 mM, 2-CHX-1 caused a dose-dependent increase in the amount of specific [ $^3H$ ]FMLP bound after 5 min at 37°C. In five binding experiments 1 mM 2-CHX-1 increased the amount of [ $^3H$ ]FMLP bound by 0.5- to 2.9-fold. There was no effect of the drug on nonspecific binding. The dose causing 50% enhancement of binding ( $ED_{50}$ ) was ~90  $\mu$ M, a concentration similar to the drug's  $ID_{50}$  for  $O_2^-$  generation and lysosomal enzyme release.

DEM also increased the amount of FMLP bound to PMN. 0.1  $\mu$ M FMLP (of which 25% was [ $^3H$ ]FMLP) was incubated with PMN preincubated with control buffer, 1 mM DEM, and 0.33 mM DEM. Under these conditions the number of [ $^3H$ ]FMLP counts bound rose from 938 in control cells to 3,284 counts bound to PMN treated with 1 mM DEM (one experiment).

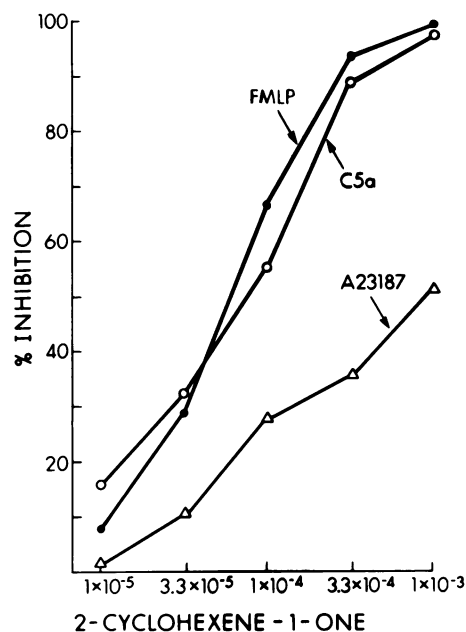


FIGURE 3 Inhibition of superoxide generation by 2-cyclohexene-1-one. Human PMN were incubated with varying concentrations of 2-CHX-1 for 30 min, washed twice, and incubated with 0.1  $\mu$ M FMLP, 10  $\mu$ l/ml C5a or 20  $\mu$ M A23187. Superoxide generation was assayed as described in the text, after 5 min for FMLP and C5a and after 15 min for A23187. Each point represents the mean of three separate experiments performed in duplicate.

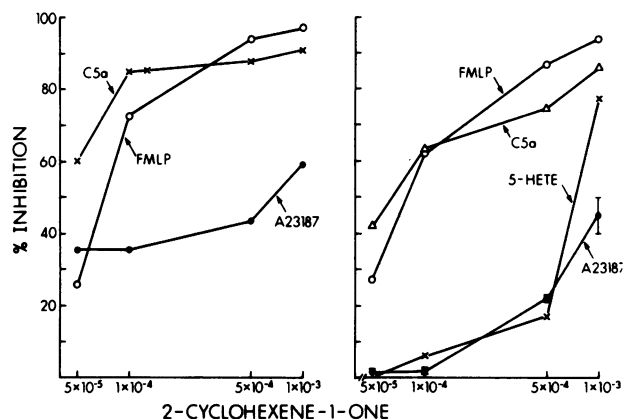


FIGURE 4 Inhibition of  $\beta$ -glucuronidase and lysozyme release by 2-cyclohexene-1-one. Human PMN were incubated with varying concentrations of 2-cyclohexene-1-one for 30 min, washed, and resuspended in complete media. PMN were stimulated with  $0.1 \mu\text{M}$  FMLP,  $10 \mu\text{l/ml}$  serum containing C5a,  $2 \mu\text{M}$  A23187, or  $5 \mu\text{M}$  5-HETE (lysozyme only) for 30 min. Experiments were performed in the absence of bovine serum albumin. Release of  $\beta$ -glucuronidase (A) or lysozyme (B) was assayed as described in the text. Each point represents the mean of three separate experiments done in triplicate. Lysozyme release in the absence of 2-CHX-1 as a percent of total cell lysozyme: zymosan  $23.4 \pm 3.0\%$ , A23187  $31.3 \pm 6.0\%$ , 5-HETE  $12.9 \pm 2.7\%$ , C5a  $27.6 \pm 5.1\%$ .  $\beta$ -Glucuronidase release in the absence of 2-CHX-1 as a percent of total cell  $\beta$ -glucuronidase: zymosan  $17.3 \pm 4.0\%$ , A23187  $8.7 \pm 3\%$ , C5a  $24.5 \pm 3.1\%$ . Enzyme release data is corrected for spontaneous release.

## DISCUSSION

The enumeration and sequencing of the dynamic series of biochemical events that characterize activated cells is of current interest. In PMN, the interaction of a variety of stimuli with the cell surface has been associated with membrane potential changes (16, 18, 19), influxes of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  (12, 20), transient elevations in cAMP levels (21, 22), and changes in protein phosphorylation (23). These events have been inferred as constituting or, at least, reflecting fundamental activation events in the pathway(s) leading to the biologic expression of chemotaxis, granule enzyme release, and  $\text{O}_2^-$  generation.

The data presented here strongly imply an important role for GSH in the activation process of human PMN. Pretreatment of PMN with 2-CHX-1 and DEM resulted in depletion of intracellular GSH levels, and a parallel inhibition of FMLP and C5a induced  $\text{O}_2^-$  generation and enzyme release, ( $\text{ID}_{50} \sim 60\text{--}90 \mu\text{M}$ ). In contrast, 2-CHX-1 was a much less potent inhibitor of those same functions stimulated by A23187 ( $\text{ID}_{50} 1 \text{ mM}$ ). The excellent correlation between depletion of GSH levels by 2-CHX-1 and the inhibition of functional responses implies a casual relationship. In addition,

TABLE III  
Effect of 2-CHX-1 on Superoxide Generation

Preincubation	Stimulus	Superoxide generation*	
		Experiment 1	Experiment 2
Control	FMLP $0.1 \mu\text{M}$	16.0	10.8
Diethyl maleate $0.1 \text{ mM}$	FMLP $0.1 \mu\text{M}$	14.6 (9.0)†	8.0
Diethyl maleate $0.33 \text{ mM}$	FMLP $0.1 \mu\text{M}$	7.5 (53.1)	3.1
Diethyl maleate $1 \text{ mM}$	FMLP $0.1 \mu\text{M}$	2.0 (87.5)	1.7
Control	A23187 $20 \mu\text{M}$	7.8	
Diethyl maleate $0.1 \text{ mM}$	A23187 $20 \mu\text{M}$	7.2 (7.7)	
Diethyl maleate $0.33 \text{ mM}$	A23187 $20 \mu\text{M}$	4.7 (39.7)	
Diethyl maleate $1 \text{ mM}$	A23187 $20 \mu\text{M}$	4.3 (44.9)	

PMN were incubated with the indicated concentrations of DEM, washed two times, and resuspended in media. Superoxide generation in response to FMLP ( $0.1 \mu\text{M}$ , 5 min) or A23187 ( $20 \mu\text{M}$  15 min) was assayed as described in the text. \* Superoxide generation is expressed as nanomoles of ferricytochrome *c* reduced per  $10^6$  PMN.  $\text{O}_2^-$  release, in the absence of stimuli, was  $\leq 0.5$  at 5 min and  $\leq 1.1$  at 15 min.

† Numbers in parenthesis are percent inhibition.

the lack of effect by a number of chemically related compounds: cyclohexene, 2-cyclohexene-1-ol, and cyclohexanone (Fig. 1) suggests that the observed effects are unlikely to be caused by physical or chemical actions unrelated to GSH metabolism. This assumption is strengthened by the parallel studies using DEM, a structurally unrelated molecule. Moreover studies of the effect of 2-CHX-1 and DEM on SH-containing compounds demonstrated that these agents were capable of combining nonenzymatically with the cysteine SH in GSH but not the SH of free cysteine or protein-bound SH groups and in the presence of PMN glutathione-S-transferase only GSH could serve as the SH donor. In addition 2-CHX-1 did not inhibit the activity of GAPDH, an enzyme that is exquisitely sensitive to SH-reactive reagents, even when this reagent was incubated with GAPDH for 90 min before the enzyme analysis, and in other studies we have shown that adenylate cyclase, which is sensitive to SH reactive compounds, is also insensitive to 2-CHX-1.<sup>4</sup> Finally, if the impairment of PMN function seen after treatment with 2-CHX-1 were caused by nonspecific toxicity one would have expected to see the same

<sup>4</sup> Wedner, H. J., J. P. Atkinson, and C. M. Fischman. Unpublished data.

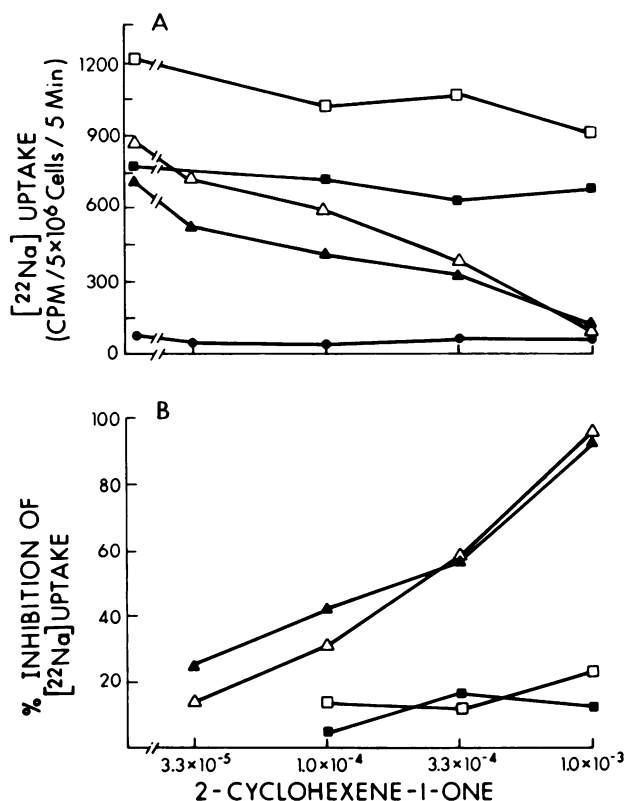


FIGURE 5 Effect of 2-cyclohexene-1-one on  $[^{22}\text{Na}^+]$  uptake by human PMN. PMN were preincubated with the indicated concentration of 2-CHX-1 for 30 min, washed twice, and resuspended in medium. PMN ( $1.8 \times 10^7$ ) were added to tubes containing 1.4  $\mu\text{Ci/ml}$   $[^{22}\text{Na}^+]$  and the appropriate stimulator in a total volume of 1.8 ml. After 5 min at 37°C, the uptake of  $[^{22}\text{Na}^+]$  was assayed in 0.5-ml portions as described in the text. Results are expressed as the mean of three separate experiments performed in triplicate. (A) Absolute uptake of  $[^{22}\text{Na}^+]$ . (B) Percent inhibition of  $[^{22}\text{Na}^+]$  uptake. ●, control; △, FMLP 0.1  $\mu\text{M}$ ; ▲, FMLP 10 nM; □, A23187 20  $\mu\text{M}$ ; ■, A23187 2  $\mu\text{M}$ .

level of impairment no matter which stimulus was used. The fact that cells treated with 2-CHX-1 (0.1 mM) have a normal or only slightly impaired response to A23187 despite a markedly impaired ( $\pm 50\%$  reduction) response to FMLP implies inhibition of the biochemical control of activation rather than impairment of the cell's ability to respond.

GSH could be critical to one or more specific steps in the activation sequence. Two lines of evidence suggest that GSH is interacting with at least one relatively early event. One, the influx of  $\text{Ca}^{2+}$  and the influx of  $\text{Na}^+$ , which appears to be responsible for depolarization of the transmembrane potential as measured by lipophilic probes and fluorescent cyanine dyes, have been shown to occur within seconds of the addition of FMLP to the cells (12, 16, 18, 19). Because these events occur within the 15- to 20-s latency period before the initiation of  $\text{O}_2^-$  generation and lysozyme release,

the increased cation fluxes are thought to reflect early activation events. If this is true, then 2-CHX-1 and DEM which inhibit the FMLP-stimulated influxes, are likely to be acting at an even earlier step. Two, despite the fact that 2-CHX-1 causes a decrease in FMLP-induced responses, GSH depletion is associated with enhancement of  $[^3\text{H}]$ FMLP binding (see below). Thus, GSH is clearly affecting directly or indirectly, the locus of the cell membrane receptor whose interaction with FMLP must be the first step in the sensory transduction mechanism of the cell.

The possibility that depressed GSH might decrease the binding of FMLP to its surface receptor was explored. Instead there was an increase in  $[^3\text{H}]$ FMLP binding when GSH levels were depressed. This appeared to be caused by an increase in the number of receptors. The reason for this increase in receptors is not clear; it may be that depletion of GSH allowed receptors that were inaccessible to FMLP to become accessible. Other maneuvers such as preincubation of cells with several aliphatic alcohols has also been reported to cause an increase in the number of available receptors for FMLP (24). The increase in FMLP (and by analogy, C5a) receptors cannot account directly for the results demonstrated here. The effects of decreased GSH are seen at supraoptimal doses of FMLP, doses that would be expected to produce the same percentage of receptor occupancy as an optimal dose of FMLP in the absence of 2-CHX-1. The functional data do suggest, however, that the newly appearing FMLP receptor sites are unable to transmit the pertinent biochemical signal to the interior of the PMN.

Although clearly not the only interpretation, the above hypothesis may explain the relative inefficacy

TABLE IV  
Effect of 2-CHX-1 on  $[^{45}\text{Ca}^{2+}]$  Uptake

Stimulus	$[^{45}\text{Ca}^{2+}]$ uptake		
	0	1 mM	0.1 mM
cpm/5 $\times 10^6$ cells/5 min			
Control	236 $\pm$ 33	222 $\pm$ 17	181 $\pm$ 85
FMLP, 0.1 $\mu\text{M}$	1,356 $\pm$ 162	490 $\pm$ 165	1,015 $\pm$ 225
FMLP, 10 nM	1,021 $\pm$ 220	255 $\pm$ 97	721 $\pm$ 115
A23187, 20 $\mu\text{M}$	5,276 $\pm$ 993	5,305 $\pm$ 1,247	5,716 $\pm$ 1,849
A23187, 2 $\mu\text{M}$	4,612 $\pm$ 508	4,503 $\pm$ 249	5,724 $\pm$ 1,446

PMN were preincubated with the indicated concentration of 2-CHX-1 for 30 min at 37°C, washed twice, and resuspended in medium. Experiments were performed in a total volume of 1.8 ml containing  $1.8 \times 10^7$  cells and  $[^{45}\text{Ca}]$ Cl<sub>2</sub> 1.4  $\mu\text{Ci/ml}$ . After a 5-min incubation with the appropriate stimulus,  $[^{45}\text{Ca}^{2+}]$  uptake was assayed in 0.5-ml portions of the cell suspensions as described in the text. The results represent the mean  $\pm$  SD for three separate experiments each performed in triplicate.

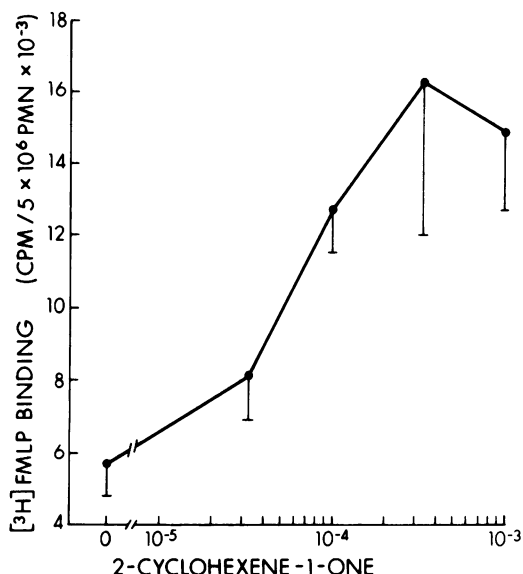


FIGURE 6 Effect of 2-cyclohexene-1-one on  $[^3\text{H}]$ FMLP binding to human PMN. Human PMN were incubated with varying concentrations of 2-CHX-1 for 30 min, washed twice, and resuspended in complete media.  $[^3\text{H}]$ FMLP,  $0.1 \mu\text{M}$  was added, and bound  $[^3\text{H}]$ FMLP separated as described in the text. Specific binding was calculated by subtracting counts bound to cells in the presence of a 250-fold excess of unlabeled FMLP. Each point represents the mean  $\pm$  SD of four separate experiments performed in triplicate.

of GSH depletion on A23187-induced functions. A23187 is lipophilic and would be expected to transport  $\text{Ca}^{2+}$  by dissolving in the hydrophobic regions of the membrane irrespective of specific membrane receptors. Moreover, A23187, by transporting  $\text{Ca}^{2+}$ , may actually bypass the early GSH-dependent step(s) that appear to involve the functional integrity of surface receptors.

A number of studies have demonstrated that maintenance of adequate GSH levels is necessary for the integrity of the external plasma membrane (25). Whether this is related to GSH itself or to alterations in the hexose monophosphate shunt or the NADP/NADPH ratio is not clear. The present study suggests that a relative decrease in plasma membrane integrity is not the cause of the inhibition of PMN function seen with 2-CHX-1 or DEM. As mentioned above these reagents are nontoxic at concentrations that depress GSH levels to 10% or less of their normal value. In addition there is no leak of either LDH or of GSH from cells treated with 1 mM 2-CHX-1 for 30 or 60 min.

To complete the case for an important role for GSH in PMN activation it would be useful to demonstrate that repletion of intracellular GSH resulted in a return of normal PMN function. Two efforts were made to demonstrate this point; unfortunately, both attempts failed. When PMN were incubated with exogenous

GSH, the GSH did not enter the cells. This is consistent with findings in other cell types (26). An attempt was also made to replete intracellular GSH by incubating GSH-depleted PMN with exogenous cysteine. These efforts were thwarted by the brief life span of PMN in culture. It was impossible to keep PMN in culture alive long enough to allow them to replete their intracellular GSH.

Mendelson et al. (27) demonstrated that GSH levels are depressed in PMN incubated with chemotactic stimuli. The levels were depressed rapidly after incubation of PMN with phagocyte stimuli and remained depressed for as long as 60 min. The authors suggested that the GSH was consumed in the detoxification of products of the respiratory burst. In a more recent study Voetman et al. (28) also demonstrated a decrease in reduced GSH in phagocytizing PMN. The measured decrease was, however, considerably less than that reported by Mendelson et al. (27). Nonetheless a 30% fall in GSH as observed by Voetman et al. (28) is significant and may explain the refractory state of cells that have been stimulated with chemotactic agents.

Although the early step in the activation sequence which is blocked by depressed levels of GSH is not known, one potential action should be mentioned. Work from our laboratory and others has demonstrated that 5-HETE and 5,12-di-HETE have many of the functional effects of other chemotactic stimuli (13, 29). These products of arachidonic acid metabolism are formed by the action of a specific lipoxygenase which produces 5-hydroperoxy-eicosatetraenoic acid from arachidonic acid with the subsequent reduction of the hydroperoxy to the hydroxyl group. Work with the RBL-1 cell line has demonstrated that 2-CHX-1, via its ability to decrease GSH levels, causes a marked increase in 5-hydroperoxy-eicosatetraenoic acid and prevents the formation of 5-HETE (30). If 5-HETE or 5,12-di-HETE act as common intermediates in the action of chemotactic stimuli, inhibition of HETE production by depressed GSH might inhibit the sequence at this early step.

In the present study we have used a novel method for specifically depressing human PMN intracellular GSH levels. The data demonstrate that a decrease in GSH is associated with marked inhibition of superoxide generation and granule enzyme release induced by FMLP and C5a but has much less effect on those functions stimulated by A23187 or 5-HETE. These data suggest that GSH plays an important role in the transmission of the signal for PMN activation from the exterior to the interior of the cell.

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