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ENHANCEMENT OF NEUTROPHIL RESPONSE BY SH-CONTAINING COMPOUNDS: MODULATION OF SUPEROXIDE AND HYDROGEN PEROXIDE PRODUCTION

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Abstract—Anti-inflammatory effects of SH compounds *in vivo* and their effects on lymphocytes and macrophages *in vitro* have been described, but little is known about the mechanism of action or their effects on the neutrophil. In the present study the activity of seven low molecular weight non-protein SH compounds was compared. At a concentration of 3×10^{-4} M all the compounds enhanced the activity of the HMP shunt of zymosan-stimulated neutrophils by 26–48% and that of PMA-stimulated cells by 6–44% above the control value (14.2 nmol CO_2/2.5 × 10^6 neutrophils/30 min). Pretreatment of neutrophils with SH compounds for 15 min resulted in enhanced release of O_2^- by stimulated neutrophils in all cases, with the exception of GSH, by up to 87% above that of control. These effects were largely related to the ability of the compounds to modulate the release of O_2^- and H_2O_2 by stimulated neutrophils when present in the reaction mixture. Only the compounds α-MPG and cysteine had a mild preserving effect on the intracellular GSH concentration of stimulated neutrophils. None of the compounds tested had any adverse effect on phagocytosis or killing of opsonized bacteria by the neutrophils. SH compounds may protect sensitive SH groups of functional proteins by providing an easily accessible source of oxidizable SH groups in times of high oxidative stress, and their ability to interact with oxygen products could in part explain their anti-inflammatory properties.

Free sulphydryl (SH) groups are highly reactive species with important functions in many biological processes. Thus, the SH groups of intracellular and plasma membrane proteins and soluble thiols are essential for neutrophil responses to stimulation and subsequent maintenance of function [1–5]. These groups are sensitive to oxidative attack and, if oxidized, neutrophil responsiveness is reduced. During phagocytosis these cells exhibit a rise in non-mitochondrial respiration and can generate large amounts of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and possibly other highly reactive oxygen radicals [6–8]. Consequently, the neutrophil and the surrounding environment can be under heavy oxidative stress, potentially resulting in depletion of SH groups of structurally and functionally important proteins. Reactive oxygen metabolites as potential mediators of cell and tissue injury have received increasing attention in recent years [9–11]. A variety of antioxidants and free-radical traps used *in vivo* have been shown to have anti-inflammatory properties and protective effect against oxidative inhibition of neutrophil function *in vitro* [12–15]. In addition, a number of low molecular weight non-protein thiols, such as penicillamine, cysteine, acetyl cysteine and mercaptopropionyl glycine (α-MPG), have been used in the treatment of inflammatory and drug-induced diseases in which free radicals, including oxygen metabolites, have been implicated as mediators of tissue injury [16–19]. The precise mechanism of action of some of these drugs still remains unclear and their effects on neutrophil oxygen metabolism have not been extensively studied.

In the present study we have compared the effects of seven different SH containing compounds on the hexose monophosphate (HMP) shunt activity, O_2^- and H_2O_2 production, intracellular glutathione (GSH) levels and bactericidal activity of normal human neutrophils.

MATERIALS AND METHODS

Penicillamine, cysteine and reduced glutathione were obtained from Sigma (Sigma Chemical Company, Poole, Dorset, U.K.) and SH compounds mercaptopropionyl glycine (α-MPG), hydroxyphenyl mercaptopropionyl thiazolidine carboxylic acid (HMTC), mercapto methylpropanoyl cysteine (MMPC) and mercaptopropionyl histidine (MPH) were kindly provided by Santen Pharmaceutical Company (Osaka, Japan).

Isolation and purification of neutrophils

Neutrophils were isolated from normal human venous blood by dextran (mol. wt. = 150,000) sedimentation of erythrocytes followed by Ficoll–Triosil (density = 1.077 g/ml) gradient centrifugation at 450 *g*. The cell pellet was washed twice with Hank's balanced salt solution (HBSS) at pH 7.4 without antibiotics and containing 0.1% bovine serum albumin, 20 mM Hepes and 5 U/ml preservative-free heparin. The residual erythrocytes were lysed

with distilled water for 20 sec and the neutrophils resuspended in HBSS to a final concentration of 1×10^7/ml. More than 95% of cells were viable as assessed by exclusion of trypan blue stain and release of lactate dehydrogenase.

Assay of $O_2^{\cdot-}$ and H_2O_2 production

Superoxide dependent ferricytochrome *c* reduction was measured by the multiwell technique of Pick and Mizel [20] modified to allow measurements to be made on neutrophils in suspension. Reaction mixtures in Mictotiter plates contained 1.25×10^5 neutrophils, 230 μM cytochrome *c* and 250 μg of opsonized zymosan or 4 nM phorbol myristate acetate (PMA) in a total volume of 150 μl HBSS per well. The reactions were performed in triplicate and control wells included neutrophils without opsonized zymosan in the presence or absence of SH compounds to be tested, and wells containing cytochrome *c* alone. Plates were incubated at 37° on a Microtiter plate shaker and the absorbance at 550 nm wavelength measured every 10 min on an automated Microelisa reader (Dynatech Laboratories Ltd., Sussex, U.K.) with the reference wavelength set at 490 nm. The results were expressed in nmol cytochrome *c* reduced per 1.25×10^5 neutrophils after subtraction of background.

Measurement of hydrogen peroxide released by stimulated neutrophils was performed by an assay essentially as described above except that the reactions were performed in 10 mM potassium phosphate buffer at pH 7 containing phenol red as the H_2O_2 trapping reagent and 4 nM PMA as the stimulus. At desired time intervals the pH in the wells was brought to 12.5 by the addition of 10 μl 1M NaOH in order to eliminate changes in the absorbance of phenol red due to its behaviour as a pH indicator [21]. The absorbance was then measured at 600 nm wavelength and the results expressed as nmol H_2O_2 released per 1.25×10^5 neutrophils as calculated from the H_2O_2 standard curve.

Hexose monophosphate shunt activity

The release of $^{14}CO_2$ from 1-^{14}C-glucose by resting and zymosan- or PMA-stimulated neutrophils was measured using the method described by Roberts *et al.* [22], with some minor modifications. The reaction mixtures in multiwell plates in a total volume of 500 μl HBSS contained 2.5×10^6 neutrophils, 0.2 μCi of 1-^{14}C-glucose (sp. act. = 3.94 mCi/mmol) and 150 μg of opsonized zymosan or 4 nM PMA. The wells were sealed with 1.5 mm thick glass fibre filters (2.1 cm diameter) impregnated with 100 μl Hymine hydroxide as the CO_2 trapping reagent and plates incubated at 37° on a Microtiter plate shaker. The reactions were terminated at the desired time intervals by the addition of 500 μl of 1M HCl to each well and the incubation continued for a further 30 min on the bench. The amount of $^{14}CO_2$ trapped in duplicate filters was estimated by scintillation counting and the results expressed as nmol CO_2 liberated from glucose per 2.5×10^6 neutrophils.

Phagocytosis and killing of bacteria

The phagocytosis and killing of opsonized *E. coli* O110 by the neutrophils was assayed using a modification of the method described for *Candida albicans* [23]. Assays were performed in Microtiter plates, each well containing in a final volume of 100 μl: 6.25×10^5 *E. coli* previously opsonized for 10 min in 10% normal serum and 1.25×10^5 neutrophils to give a ratio of *E. coli* to neutrophils of 5:1. After 10 and 20 min incubation at 37° on a Microtiter plate shaker 167 μCi of ^{3}H uridine was added to each well (sp. act. = 40 Ci/mmol) followed by 100 μl HBSS to wells in which phagocytosis was to be assessed, and 50 μl deoxycholate followed by 50 μl DNAse to wells used for estimation of the killing capacity of the neutrophil. The plates were then incubated for a further 30 min without shaking and the contents of the wells collected on to filter papers using a 12-channel cell harvester. The amount of radioactivity collected on the filters was determined by scintillation counting and the phagocytic (PI) or killing (KI) index calculated from the following formula:

$$\text{PI or KI} = \left(1 - \frac{\text{cpm } \textit{E. coli} + \text{neutrophils}}{\text{cpm } \textit{E. coli} \text{ alone}}\right) \times \textit{E. coli}/\text{neutrophil ratio.}$$

GSH assay

The fluorometric technique of Hissin and Hilf [24] was used. This assay is based on the reaction of GSH with *O*-phthaldaldehyde at pH 8 to yield a fluorescent product that could be activated at 350 nm with an emission peak at 420 nm wavelength. Aliquots (500 μl) of neutrophil suspension (1×10^7/ml) were washed twice with 0.1 M potassium phosphate buffer (pH 8) containing 5 mM EDTA and the cells were lysed by the addition of 2.5 mg of deoxycholate in 200 μl of buffer followed by 200 μl of metaphosphoric acid (25% aq.). The resultant precipitate was removed by centrifugation at 12,000 *g* for 1 min and the GSH content assayed in 300 μl of supernatant. The results were expressed in nmol GSH/10^7 neutrophils as calculated from the GSH standard curve. The specificity of the assay for GSH was ascertained by the inclusion of 40 μg of each of the SH compounds tested in the GSH standards.

Oxidation of SH compounds

Aliquots (1 ml) of different SH compounds at a concentration of 3 μmol/ml i.u. phosphate-buffered saline (pH 7.4) were each treated with 6 μmol of H_2O_2 for 60 min at 37°. This was followed by the addition of 15 units of catalase to each solution and incubated for a further 60 min to remove the residual H_2O_2. The oxidation of the SH groups was ascertained by DTNB reactivity. H_2O_2 (6 mM) treated with catalase in the absence of SH compounds served as the control.

RESULTS

HMP shunt activity

The effect of seven SH-containing compounds on the metabolism of 1-[^{14}C] glucose via the HMP shunt by zymosan- or PMA-stimulated neutrophils is shown in Table 1. Incubation of neutrophils with opsonized zymosan in the presence of SH compounds at a concentration of 3×10^{-4} M enhanced the HMP shunt activity above that of control cells stimulated

Table 1. HMP shunt activity (nmol $CO_2/2.5 \times 10^6$ neutrophils)

	15 min			30 min		
	HBSS	Zymosan	PMA	HBSS	Zymosan	PMA
Control	1.7	9.5	6.3	2.1	14.2	7.8
MMPC						
1.5×10^{-4} M	—	—	—	—	17.5	—
3.0×10^{-4} M	1.2	17.3	5.8	1.6	18.9	9.6
HMTC						
1.5×10^{-4} M	—	—	—	—	15.1	—
3.0×10^{-4} M	1.3	13.0	5.2	1.8	20.3	9.5
MPH						
1.5×10^{-4} M	—	—	—	—	14.3	—
3.0×10^{-4} M	1.5	17.3	—	1.9	18.0	—
PEN						
1.5×10^{-4} M	—	—	—	—	14.0	—
3.0×10^{-4} M	1.2	19.1	5.4	2.2	20.1	11.2
GSH						
1.5×10^{-4} M	—	—	—	—	15.3	—
3.0×10^{-4} M	1.6	13.9	6.1	2.0	20.5	8.3
CYS						
1.5×10^{-4} M	—	—	—	—	14.8	—
3.0×10^{-4} M	1.3	19.9	5.5	2.0	21.1	10.3
α-MPG						
1.5×10^{-4} M	—	—	—	—	13.8	—
3.0×10^{-4} M	1.6	19.4	6.5	2.2	19.8	9.2

in the absence of these compounds. The increase in activity after 30 min of incubation ranged from 26% in the presence of MPH to 48% with cysteine. Similar effects were seen in case of the PMA-stimulated neutrophils. Increasing the concentration of the compounds to 6×10^{-4} M in the reaction mixture did not produce further enhancement in the HMP shunt activity.

At the lowest concentration used (1.5×10^{-4} M) only MMPC produced a significant enhancement in the activity of the shunt in zymosan-stimulated neutrophils. The stimulatory effect was most pronounced in the early stages of neutrophil activation by zymosan (15 min) whereby all compounds tested, with the exception of GSH, induced an early optimal CO_2 accumulation. Stimulation with opsonized zymosan or PMA of washed neutrophils, previously incubated for 15 min with individual SH compounds at a concentration of 3×10^{-4} M, yielded similar results to those when the cells were stimulated in the presence of the compounds. If, however, the oxidized form of the SH-containing compounds was used at the same concentration, no enhancement effect was observed in either zymosan- or PMA-stimulated cells. None of the compounds, whether in reduced or oxidized form, had any effect on the HMP shunt activity of resting neutrophils.

Production of O_2^- *and* H_2O_2.

The results shown in Fig. 1 demonstrate the effects of the SH compounds on the kinetics of O_2^--dependent cytochrome *c* reduction by stimulated neutrophils. In the absence of SH compounds neutrophils stimulated with opsonized zymosan reduced 6.56 ± 0.4 nmol cytochrome *c*/1.25×10^5 neutrophils in 50 min. In the presence of SH compounds at concentrations of 3×10^{-4} M, with the exception of GSH, there was an apparent inhibition of O_2^- release by stimulated neutrophils ranging from only 20% in the presence of HMTC to 100% in the case of MMPC. Similar effects were seen when cells were stimulated with phorbol myristate acetate (PMA) and the most effective inhibition was again achieved in the presence of MMPC, followed by penicillamine, cysteine and α-MPG. In the presence of HMTC and MPH the rate of O_2^- production remained unaffected for up to 30 min incubation but was significantly reduced thereafter. The reduction of cytochrome *c* by PMA-stimulated neutrophils in the presence of SH compounds was effectively inhibited by superoxide dismutase only during the first 30 min of incubation for MPG, cysteine and penicillamine and 20 min for MMPC. Zymosan-stimulated cells produced considerably less O_2^- and cytochrome *c* reduction in the presence of these four compounds was effectively inhibited by superoxide dismutase during the whole course of the reaction.

The inhibitory effect on O_2^- production by the SH compounds was no longer apparent if neutrophils were preincubated for a short period of time with 3×10^{-4} M concentration of individual SH compounds and then washed with HBSS prior to stimulation. Rather than being inhibitory, this treatment enhanced the response of neutrophils to opsonized zymosan (Fig. 2), with varying degrees of efficacy in all cases, with the exception of GSH. This enhancement effect did not appear to be related to the ability of compounds to suppress O_2^- release when present in the reaction mixture. Preincubation of neutrophils in MPH induced the cells to reduce almost twice the amount of cytochrome *c* after stimulation when compared to control cells (3.15 ± 0.52 and 5.91 ± 0.49 nmol respectively). Similar effects were observed when PMA was used as the stimulus for these cells; however, the enhancement was not so pronounced largely due to the already fast kinetics of O_2^- production in untreated cells.

In a separate series of experiments, neutrophils

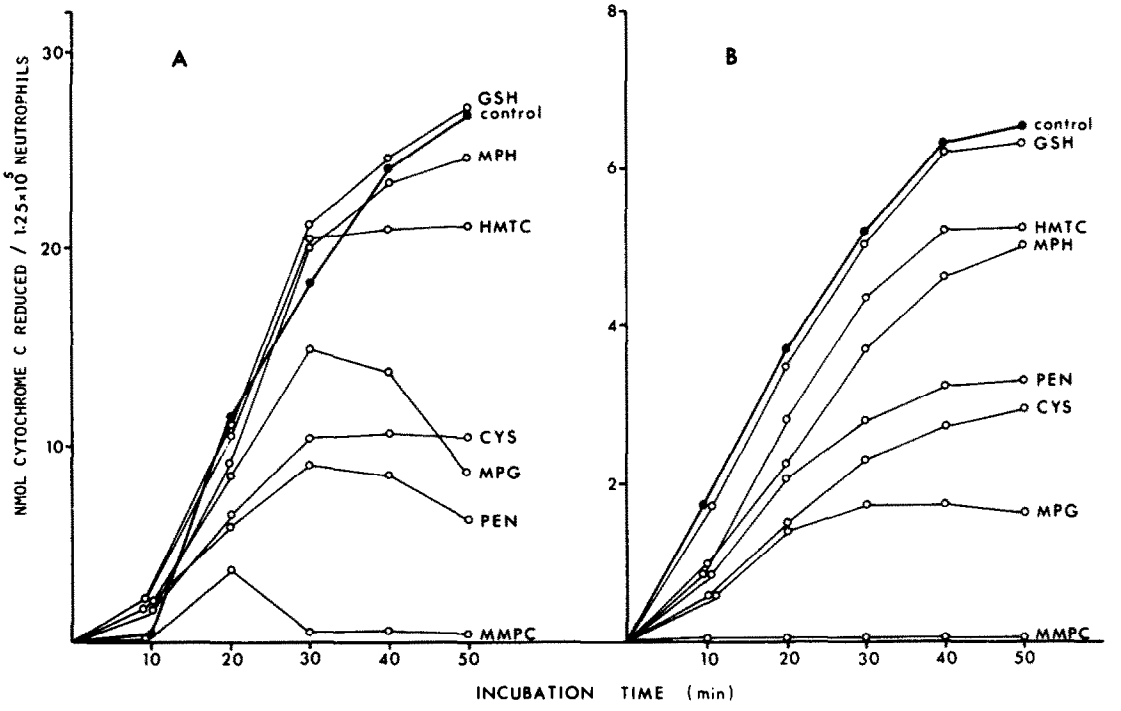

Fig. 1. Effect of SH compounds on O_2^- production by (A) PMA or (B) zymosan-stimulated neutrophils. Key: (●) neutrophils stimulated in the absence of SH compounds; (○) neutrophils stimulated in the presence of 3×10^{-4} M SH compounds.

preincubated with 4 nM PMA for 30 min and then washed, were used for the study of O_2^- production in response to a second, different stimulus. This treatment reduced the response of neutrophils to opsonized zymosan by 95% when compared to the control value. If, however, SH compounds (3×10^{-4} M) were included in the preincubation mixtures with the neutrophils and PMA, there was a marked improvement in their subsequent response to opsonized zymosan (Fig. 3). Compounds MMPC and GSH had no effect in this assay.

When the effect of SH compounds on the production of H_2O_2 by PMA-stimulated neutrophils was studied, a different pattern emerged, as shown in Fig. 4. α-MPG, HMTC, MMPC and, to a lesser extent, penicillamine produced a concentration-depedent inhibition of H_2O_2 release by stimulated neutrophils but no effect was detected on the resting cells. MPH had only a slight effect on H_2O_2 release at 3×10^{-4} M concentration, but increasing the concentration to 6×10^{-4} M produced a significant reduction in the amount of H_2O_2 detected. The degree to which H_2O_2 release was inhibited was unrelated to the effect of these compounds on the O_2^- production, suggesting that the SH compounds may have different affinities for the two oxygen products. Cysteine and GSH had no effect in this assay system.

Intracellular GSH content and bactericidal activity

Activation of neutrophils with opsonized zymosan leads to 50% reduction in the intracellular GSH content after the 40 min incubation period used (from 12.2 to 6.1 nmol/10^7 cells). Despite the reduced levels of O_2^- and H_2O_2 released by stimulated neutrophils in the presence of some of the SH compounds, the loss of intracellular GSH was not prevented. However, cysteine and MPG at concentrations of 6×10^{-4} M had some preserving effect on GSH in that the levels were reduced to only 68% of the control value in their presence. Whereas MPG was very effective in blocking O_2^- and H_2O_2 release, cysteine was only effective in inhibiting O_2^- release. The GSH content of resting neutrophils was not affected by treatment with SH compounds.

Preincubation of neutrophils with SH compounds or their inclusion in the reaction mixture in concentrations up to 6×10^{-4} M had no effect on either the phagocytosis or killing of *E. coli* by neutrophils. After 30 min of incubation the phagocytic and killing index values, in the presence of various SH compounds, ranged from 4.9 to 5.0 and from 4.9 to 5.1 respectively and none were significantly different from the control values of 5.0 ± S.D. 0.15 for the phagocytic index and 5.1 ± 0.15 for killing index.

DISCUSSION

Neutrophils can generate appreciable amounts of toxic oxygen products and considerable evidence now exists to suggest that auto-oxidation is the basis for altered neutrophil function and is a process involved in limiting neutrophil response to a stimulus [11, 25, 26]. Earlier studies have shown that SH compounds such as cysteine and mercaptopropionyl glycine (α-MPG), both effective free-radical trapping agents, can potentiate neutrophil locomotion *in vitro* [15, 27, 28] by, as yet, unidentified mechanisms. The results with regard to penicillamine are conflict-

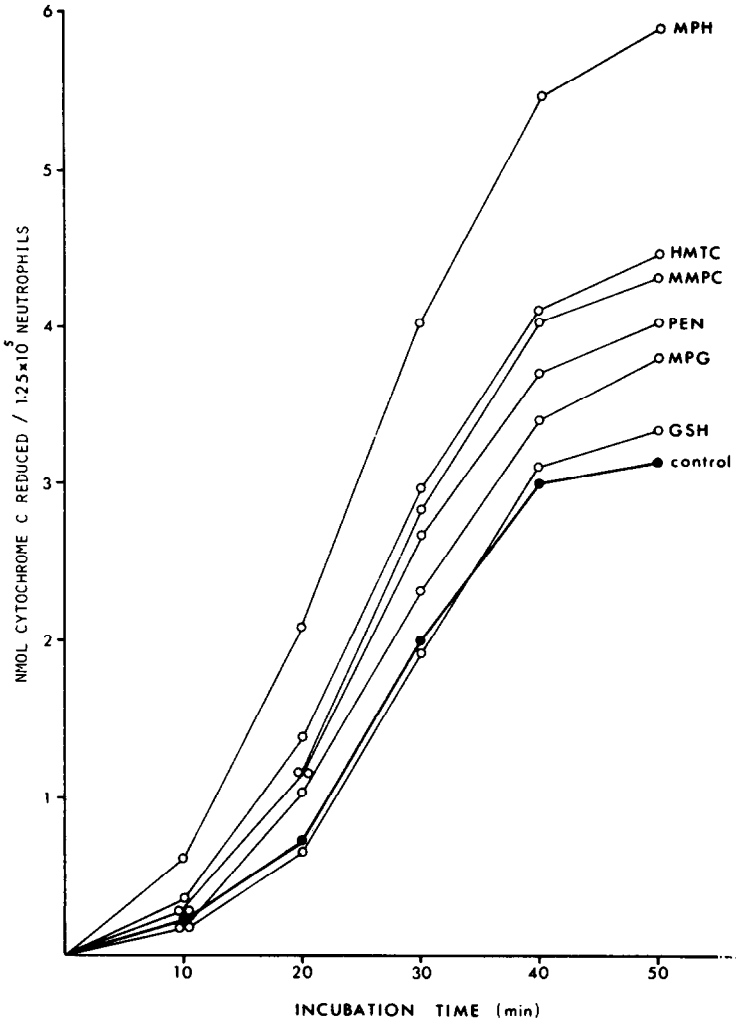

Fig. 2. O_2^- production by zymosan-stimulated neutrophils pretreated with SH compounds. Key: (●) neutrophils preincubated in HBSS at 37° for 15 min and washed with HBSS before stimulation; (○) neutrophils pretreated with 3×10^{-4} M SH compounds.

ing showing a spectrum of activity ranging from inhibition to stimulation of neutrophil locomotion [29–31]. In the present study the enhancing effect of SH compounds on the stimulus-dependent activation of the HMP pathway of glucose metabolism in the human neutrophil appears to be, initially, by a common mechanism. The activity of this pathway reflects increased turnover of NADPH attributable in greater part to the requirements of the neutrophil oxidase system generating the O_2^- radical and, to a lesser extent, to the activity of the glutathione cycle responsible for detoxification of H_2O_2. The function of the neutrophil oxidase is dependent on free SH groups [32] and is therefore potentially susceptible to oxidative attack by its own products. Considerably less O_2^- was detected when neutrophils were activated in the presence of SH compounds than in their absence. It is unlikely that production of this free radical is inhibited since this would also lead to reduced activity of associated metabolic pathways such as the HMP shunt and also possibly reduce the killing of bacteria, which was not the case in our study. However, the removal of the produced O_2^- would reduce the oxidant stress on the neutrophil, thus protecting the oxidase system and allowing its prolonged function. This in turn would lead to increased turnover of NADPH with consequent enhancement of the HMP shunt activity as observed in our experiments. Oxidized thiols, however, had no effect on the HMP shunt activity of either resting or stimulated neutrophils. This suggests that NADPH- or GSH-mediated reduction of SH compounds, oxidized during cell activation, is not the mechanism by which these compounds enhance the HMP shunt activity of neutrophils.

Hydrogen peroxide is generated predominantly from spontaneous or enzymic dismutation of O_2^- [11] and it follows that quenching of O_2^- or inhibition of its synthesis would lead to a reduction in detectable levels of H_2O_2 as well. In the present study the reduction in the levels of O_2^- generated did not necessarily lead to a proportional decrease in the detectable H_2O_2. This was most striking in the case of cysteine which reduced the O_2^- release by as much as 30% without having any effect on H_2O_2. There is no obvious explanation for this discrepancy but it

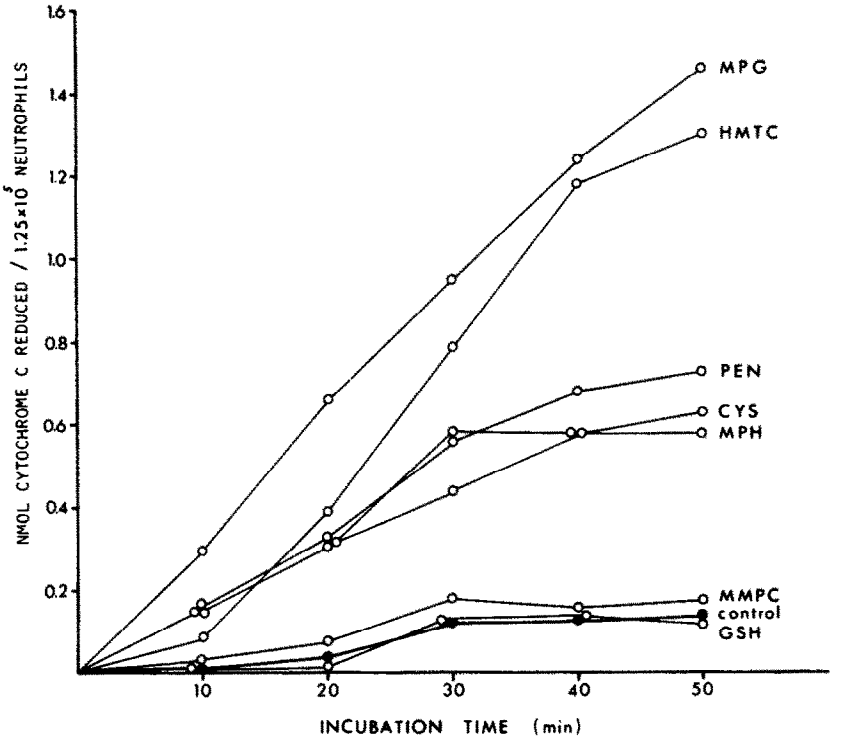

Fig. 3. O_2^- production by zymosan-stimulated neutrophils pretreated with PMA in the presence of SH compounds. Key: (●) neutrophils pretreated for 30 min at 37° with 4 nM PMA and washed with HBSS: (○) neutrophils pretreated with PMA in the presence of 3×10^{-4} M SH compounds.

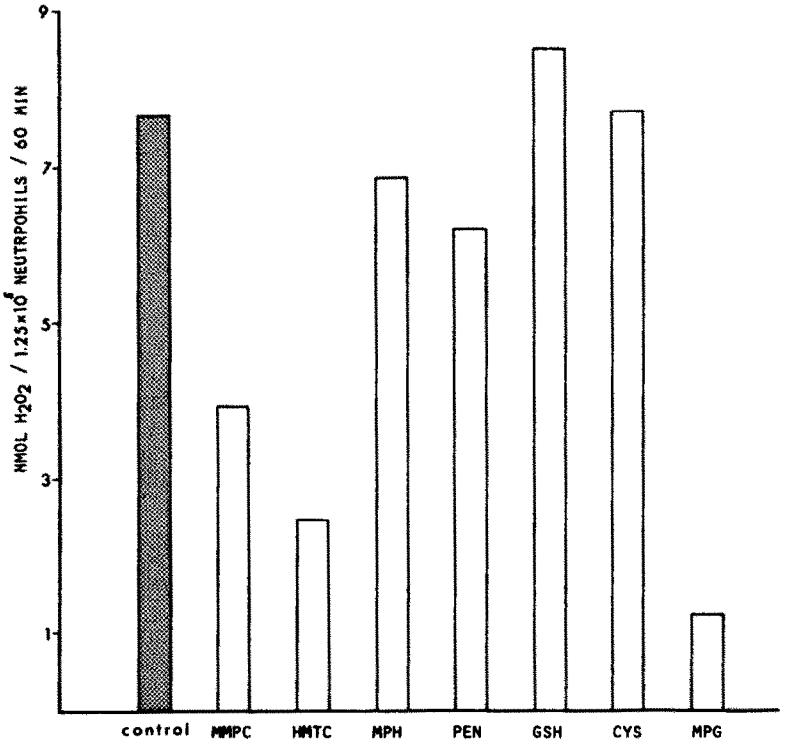

Fig. 4. Effect of SH compounds on H_2O_2 production by PMA-stimulated neutrophils. Key: (▨) neutrophils stimulated with 4 mM PMA in the absence of SH compounds; (□) neutrophils stimulated in the presence of 3×10^{-4} M SH compounds.

may be possible that cysteine plays a role in the dismutation of O_2^-. Conversely, HMTC had only a small effect on O_2^- release whereas it was very effective in reducing the levels of H_2O_2 detected in the system. Taken together these results suggest that the SH compounds tested have varying affinities towards different oxygen metabolites, presumably related to the reactivity of the SH group on the molecule and, thus, afford different levels of protection to the neutrophil.

With the possible exception of α-MPG and cysteine, extra-cellularly added SH compounds do not appear to be involved in the GSH-dependent detoxification of oxygen products. It is difficult to explain the enhanced release of O_2^- from neutrophils preincubated with the SH compounds and then activated in their absence. It may be significant that GSH, a thiol that cannot cross the plasma membrane, was the least active compound throughout the study. Pretreatment of neutrophils with SH compounds other than GSH, which may be more permeable to the cell, could therefore provide the neutrophil with an additional store of easily accessible SH groups. Although their role may not be primarily in detoxification reactions, they may be utilized for the formation of mixed disulphides with sensitive SH groups of functional proteins in times of high oxidant stress or for regeneration of SH groups after the removal of the oxidizing species. These suggestions are, to some extent, substantiated by experiments in which PMA-induced inhibition of neutrophil response to opsonized zymosan was partially prevented or reversed by the introduction of SH compounds into the preincubation mixtures. Further work is in progress to establish the precise mechanism of action of these compounds and whether mixed disulphide formation indeed does occur.

The ability of SH compounds to suppress the release or the activity of the reactive oxygen metabolites may in part explain the anti-inflammatory properties of compounds such as penicillamine and α-MPG and also suggests a mechanism by which they and other SH compounds may potentiate the function of cells involved in the immune system. Mercaptopropionyl derivatives such as MMPC, MPH, HMTC and α-MPG produced a broad spectrum of activities in our study and, in the majority of assays, proved to be the most effective agents. It is noteworthy that the oxygen metabolite scavenging properties do not interfere with the major neutrophil function of bacterial destruction. This would suggest considerable therapeutic value for these compounds and may explain their beneficial effects, which have been reported in a number of clinical trials [33–36]. Most of these relate to the use of penicillamine in rheumatoid arthritis, chronic active hepatitis and primary biliary cirrhosis, but trials of α-MPG also demonstrate the therapeutic potential of a different SH-containing compound in patients with chronic active hepatitis. Recently it has been suggested that oxygen free radical reactions may also mediate the more permanent features of advanced alcoholic liver disease [37] which would suggest that SH compounds may also have beneficial effects in these patients. The use of penicillamine, however, is associated with a number of side-effects [38], especially in patients with chronic liver disease [39], which are not as prevalent when a different SH compound, α-MPG, is used [40]. The possible diverse mechanism of action of different thiols suggests the need for further clinical trials with novel SH-containing drugs, such as the ones used in this study, in order to optimize the beneficial effects and reduce the risk of adverse reactions.

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