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INHIBITION OF NEUTROPHIL FUNCTION BY HYDROGEN PEROXIDE

EFFECT OF SH-GROUP-CONTAINING COMPOUNDS

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Abstract—Stimulated neutrophils generate appreciable amounts of hydrogen peroxide (H_2O_2) which may be responsible for auto-oxidative injury and damage to adjacent cells. In the present study we describe inhibitory effects of H_2O_2 on neutrophil phagocytosis, bactericidal activity and associated metabolic processes as well as the effect of non-protein SH-compounds on H_2O_2-treated cells. Preincubation of neutrophils with low concentrations of H_2O_2 (1 μmoles/5 $\times$ 10^6 cell) results in delayed phagocytosis of *Escherchia coli*, which returns to normal levels in the later stages of incubation, while the activity of the HMPS and the production of O_2^- and H_2O_2 remain unaffected. Bactericidal activity of the cells was more sensitive to peroxide treatment and even at low concentrations H_2O_2 induced some inhibition (12.2%) of neutrophils' capacity to kill *E. coli*. Increasing the concentrations of H_2O_2 in the preincubation mixtures resulted in a progressive decline in the neutrophils phagocytic and killing capacity for *E. coli* and was accompanied by inhibition of HMPS activity and the release of granule enzymes but not of O_2^- or H_2O_2. The H_2O_2/O_2^- molar ratio of peroxide-treated cells was elevated by up to 26.7% and this was followed closely by the reduction in the intracellular levels of reduced glutathione (GSH). Incubation of H_2O_2-treated neutrophils with all five SH-compounds used in the study resulted in the improvement of the phagocytic capacity of the cells. Improvement of the bactericidal capacity and degranulation responses of H_2O_2-treated neutrophils was achieved by incubation with cysteine, penicillamine, α-MPG and MMPC but not GSH. Stimulus-dependent H_2O_2 production by H_2O_2-treated cells, the H_2O_2/O_2^- molar ratio and the intracellular levels of GSH remained unaltered after treatment with SH-compounds. The data shows that SH-compounds, in addition to their anti-inflammatory properties, also have the ability to reverse the oxidant-induced inhibition of neutrophil function, a property of potential therapeutic significance.

Neutrophils, the body's major defence against bacterial infection, possess a number of toxic systems that are dormant when the cell is at rest but which can be activated when the need arises to destroy invading micro-organisms [1]. Although the bactericidal systems of the neutrophil are normally confined to the phagocytic vacuole, leakage or secretion of toxic agents into the extracellular space can occur with a potential for cell and tissue injury. During the process of neutrophil activation considerable amounts of oxygen metabolites are produced by the cells, particularly superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [2, 3], and numerous studies have described their cytotoxic potential [4]. Neutrophils are also susceptible to the toxic effects of H_2O_2 they generate and although detoxification systems such as the glutathione cycle [5] and catalase [6] are available within the cell, oxidative injury to the neutrophil can take place [7–9].

In our earlier study we demonstrated that low molecular weight non-protein SH-group-containing compounds, added extracellularly, can enhance neutrophil responses to a stimulus *in vitro* and that this effect was related to the ability of the thiols to interact with the oxygen metabolites produced by the cell [10]. In the present paper we describe the inhibition by H_2O_2 of neutrophil phagocytosis and killing of *E. coli*, oxygen metabolite production, degranulation, activation of the hexose monophosphate shunt (HMPS) and the effect of SH-group-containing compounds on the H_2O_2-treated cells.

MATERIALS AND METHODS

Penicillamine (PEN), cysteine (CYS) and reduced glutathione (GSH) were obtained from Sigma (Sigma Chemical Co., Poole, Dorset) and SH-compounds mercaptopropionyl glycine (α-MPG) and mercaptomethylpropionyl cystein (MMPC) were kindly donated by Santen Pharmaceutical Company (Osaka, Japan).

Preparation of neutrophils. Neutrophils were isolated from heparinized venous blood, by dextran (average molecular wt 150,000) sedimentation of erythrocytes and Ficoll–Hypaque gradient centrifugation as previously described [10]. A working suspension was prepared in Hank's balanced salt solution (HBSS) at a concentration of 1 $\times$ 10^7 cells/ml and the viability ascertained with trypan blue.

Microorganisms. Cultures of *E. coli* were grown in trypticase soya broth overnight and the washed

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cells resuspended in HBSS to a concentration of 2.5×10^8 cells/ml. Immediately prior to use in the assays bacterial suspensions were diluted to 5×10^7/ml in HBSS-containing normal serum pool at a final concentration of 10% and incubated for 10 min at 37°.

Assays of phagocytosis and killing of bacteria. The assays were performed in 96-well flat bottom tissue culture plates as described previously [10]. Bacteria (1.25×10^6) and neutrophils, at a bacteria/neutrophil ratio of 20:1, were mixed in a final volume of 100 μl per well. The plates were incubated at 37° on a Microtiter plate shaker and at the desired time intervals 167 μCi of [^{3}H]uridine in 25 μl final volume added to all wells. This was followed by 100 μl of HBSS to wells used for estimation of phagocytosis or 50 μl of sodium deoxycholate solution (12.5 mg/ml in water) and 50 μl of DNAase (1 mg/ml) to wells used for estimation of bacterial killing. After a further 30 min period of incubation the content of wells was collected with the aid of a 12-channel cell harvester (Flow Laboratories Ltd., Irvine, Scotland, U.K.). The amount of radioactivity collected on individual filters was measured in an automatic scintillation counter (*CPM*) and the phagocytic (*PI*) and killing (*KI*) indices calculated from the following formula:

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

The percentage of ingested bacteria that have been killed was calculated from the expression: $(KI/PI) \times 100$.

Assays of O_2^- *and* H_2O_2. The superoxide-dependent reduction of ferricytochrome c was measured by a semi-automated technique described earlier [10].

Reaction mixtures in 96-well tissue culture plates contained 1.25×10^5 neutrophils, 242 μM cytochrome c and 250 μg of opsonise zymosan in a final volume of 150 l of HBSS. Plates were incubated at 37° on a Microtiter plate shaker and the absorbance of the reaction mixtures measured every 10 min using a Microelisa autoreader (Dynatech Laboratories Ltd., U.K.). The results were expressed as nmoles cytochrome c reduced per 1.25×10^5 neutrophils after subtraction of absorbance in wells containing superoxide dismutase.

Hydrogen peroxide was measured in an assay system similar to the one described above using phenol red as the peroxide trapping agent. The reactions were performed in 10 mM potassium buffer at pH 7 containing, in addition to neutrophils and stimulus, 1.2 units of horseradish peroxidase and 15 g of phenol red in a final volume of 150 μl per well. At the desired time intervals the reaction was terminated by the addition of 10 μl of 1 M NaOH and the absorbance measured at 600 nm. The concentration of H_2O_2 in the test samples (nmoles/1.25×10^5 neutrophils) was determined from the standard curve.

Activity of the hexose monophosphate shunt (HMPS). The oxidation of [^{14}C]glucose to $^{14}CO_2$ in resting and stimulated neutrophils was measured by a multiwell technique [10].

Reactions contained 2.5×10^6 neutrophils, 0.2 μCi of [^{14}C]glucose and either 150 μg of opsonized zymosan, 5 mM diamide or 5 mM methylene blue as the stimulus in a final volume of 500 μl of HBSS. The wells were sealed with 2.3 mm diameter glass-fibre filters impregnated with 100 μl of Hymine hydroxide. After a 30 min incubation on a shaker at 37° the reaction was terminated by addition of 500 μl of 1 M HCl and the incubation continued for a further 30 min. The amount of $^{14}CO_2$ collected in the filters was measured by scintillation counting and the results expressed as nmoles CO_2 generated/2.5×10^6 neutrophils.

Degranulation. The release of granule enzymes from neutrophils in response to stimulation was measured by a modification of a method described by Goldstein *et al.* [11]. The reactions were carried out in 1.5 ml microfuge cups containing 2.5×10^6 neutrophils, preincubated for 5 min with cytochalasin B (5 μg/ml) and 150 μg of opsonized zymosan as the stimulus, in a final volume of 500 μl of HBSS. The supernatants were analysed for the presence of lysosome [12], myeloperoxidase [13] and lactate dehydrogenase [14]. The results of degranulation were expressed as the percentage release of the total content.

Intracellular content of reduced glutathione (GSH). The fluorometric assay of Hissin and Hilf [15] was used. Aliquots of (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 final pellet resuspended in 300 μl of the buffer. The neutrophils were lysed with sodium deoxycholate (100 μl of 1.25 mg/ml solution) and the lysate treated with 100 μl of metaphosphoric acid (35% aq.). Supernatants were collected by centrifugation and assayed for GSH content using *o*-phthaldialdehyde. Fluorescence of solutions was measured (excitation 350 nm, emission 420 nm) and the concentration of GSH determined from the standard curve. The specificity of the assay for GSH was demonstrated in our earlier study [10]. Preincubation of neutrophils: the effects of H_2O_2 on different neutrophil functions was studied by preincubating aliquots (1 ml) of neutrophil suspension (5×10^6/ml) with different concentrations of reagent H_2O_2 for 30 min at 37°. These cells were washed twice with HBSS and then preincubated with either different concentrations of various SH compounds or HBSS alone for a further period of 30 min. Washed cells were then used in the assays of function and metabolic activity. The SH compounds were used at a concentration of 3×10^{-4} M, the concentration shown in our earlier study to have the optimal effect [10].

Presentation of results and statistical analysis. All experiments were performed in duplicate and the data presented in the form of the mean ± 1 S.D. of results obtained with neutrophils isolated from four normal subjects. The results of different treatments were compared by testing the extreme range of pair differences using the Walsh test.

RESULTS

Effect of H_2O_2 *on normal neutrophil function*

Phacocytosis and killing of E. coli. Treatment of neutrophils with H_2O_2 in the concentration range of

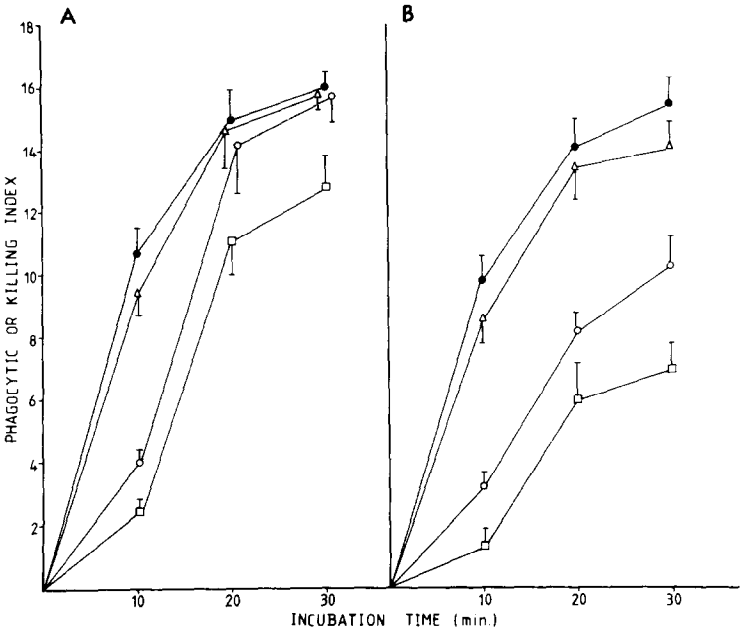

Fig. 1. Effect of treatment with different concentrations of H_2O_2 on neutrophil phagocytosis (A) and killing (B) of *E. coli*. ●, control; △, 1 μmole H_2O_2; ○, 3 μmoles H_2O_2; □, 6 μmoles H_2O_2.

0–6 μmoles/5 × 10^6 cells reduced the phagocytosis of *E. coli* in a concentration dependent fashion (Fig. 1A). Using H_2O_2 concentrations up to 3 μmoles/5 × 10^6 neutrophils in the preincubation mixtures resulted in a significant reduction of *PI* values only in the first 10 min of incubation of neutrophils with *E. coli*. Phagocytosis was reduced by 12.2 and 62.6% when neutrophils were treated with 1 or 3 μmoles of H_2O_2 respectively (control neutrophils *PI* = 10.7 ± 0.8; H_2O_2-treated neutrophils *PI* = 9.4 ± 0.7 and 4.0 ± 0.5 for 1 and 3 μmoles of H_2O_2, respectively). Continuing the incubation of reaction mixtures for a further 10 and 20 min resulted in the return of the *PI* to normal values. Using 6 μmoles of H_2O_2 in the preincubation mixtures resulted not only in further reduction of phagocytosis in the early stages of incubation (*PI* = 2.5 ± 0.3 after 10 min of incubation) but also at all subsequent time intervals studied. The *PI* values of 11.0 ± 1.0 and 12.8 ± 1.0, for the 20 and 30 min incubation, were significantly lower ($P < 0.01$) than those for the control cells (*PI* = 14.9 ± 0.9 and 15.9 ± 0.5 for the 20 and 30 min incubation periods, respectively).

The killing of *E. coli* by neutrophils appeared to be more sensitive to peroxide treatment than phagocytosis as the *KI* values were depressed considerably more than the *PI* values at all concentrations of H_2O_2 used and at all time intervals studied (Fig. 1B). The percentage of ingested *E. coli* that were killed (Fig. 2) after 10 min (90.1 ± 2.1%) and 20 min (90.5 ± 3.9%) of incubation by neutrophils treated with 1 μmole of H_2O_2 was not significantly different from that of control cells (90.7 ± 3.1% and 93.1 ± 1.6% for the 10 and 20 min incubation periods, respectively). Subsequently the

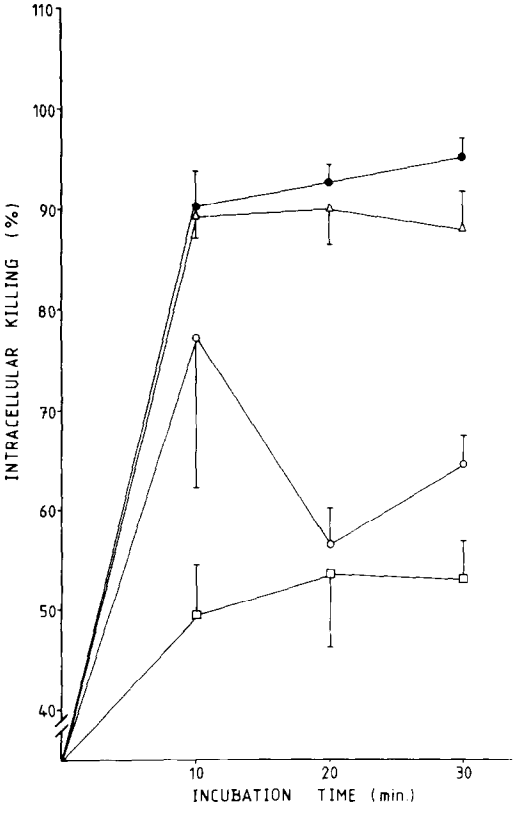

Fig. 2. Effect of treatment with different concentrations of H_2O_2 on intracellular killing of *E. coli* by neutrophils. ●, control; △, 1 μmole H_2O_2; ○, 3 μmoles H_2O_2; □, 6 μmoles H_2O_2.

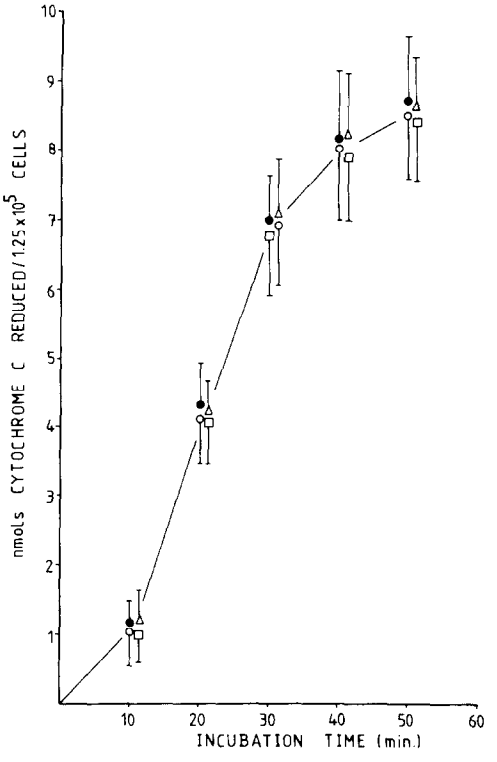

Fig. 3. Effect of treatment with different concentrations of H_2O_2 on opsonized zymosan-stimulated production of O_2^- by neutrophils. ●, control; △, 1 μmole H_2O_2; ○, 3 μmoles H_2O_2; □, 6 μmoles H_2O_2.

percentage of killed intracellular bacteria was reduced to 88.3 ± 3.2%, a value significantly lower ($P < 0.01$) than that for control neutrophils (95.6 ± 1.5%). Increasing the H_2O_2 concentration in the preincubation mixture to 3 and 6 μmoles resulted in a further decrease in the intracellular killing of *E. coli* at all incubation periods studied.

O_2 and H_2O_2 production. The production of O_2^- by neutrophils in response to stimulation by opsonized zymosan was not affected significantly by treatment of cells with up to 6 μmoles of H_2O_2 (Fig. 3). Only a 3% reduction in total and 4% in the rate of O_2^- production was detected in H_2O_2-treated neutrophils.

The production of H_2O_2 by H_2O_2-treated neutrophils in response to the same stimulus was significantly higher than in the untreated control cells (Table 1). The H_2O_2-treated cells produced up to 21% more H_2O_2 than cells preincubated in HBSS alone. There was no increase in the spontaneous release of H_2O_2 from resting cells, whether preincubated with H_2O_2 or HBSS alone. The H_2O_2/O_2^- molar ratio was significantly higher ($P < 0.01$) for H_2O_2-treated cells (1.09 ± 0.08) than for the control cells (0.86 ± 0.06).

Release of granule enzymes. The opsonized zymosan-dependent release of lysozyme and myeloperoxidase by H_2O_2-treated neutrophils was significantly reduced when compared to the control cells (Fig. 4). Preincubation of neutrophils with 1 μmole H_2O_2 had no effect but 3 μmoles of peroxide resulted in stimulus-dependent release of 13.6 ± 1.8% of lysozyme and 25.2 ± 4.1% of myeloperoxidase content of the cells. This release was further reduced to 13.2 ± 1.4 and 23.7 ± 4.5%, respectively, when neutrophils were preincubated with 6 μmoles of H_2O_2 (control value 16.2 ± 1.7% for lysozyme and 28.9 ± 1.9% for myeloperoxidase). The total content of the two enzymes remained unchanged after treatment with either concentration of peroxide.

There was no significant increase in the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) by peroxide-treated neutrophils. After preincubation of neutrophils in HBSS alone, 3.5–6% of the total LDH content of the cells was detected in the supernatant whilst neutrophils preincubated with the highest concentration of H_2O_2 used (9 μmoles/5×10^6 cells) released only 5–8% of the total LDH content (total content = 0.51 ± 0.03 IU/5×10^6 neutrophils).

HMPS activity and GSH content of neutrophils. Treatment of neutrophils with H_2O_2 in the concentration range of 1–9 μmoles/5×10^6 cells resulted in a subsequent concentration-dependent decrease in the activity of the zymosan-stimulated HMPS (Table 1). Up to 40% reduction in the activity of this pathway was achieved using 9 μmoles of H_2O_2 in the preincubation mixtures. The reduction in the activity of HMPS in H_2O_2-treated cells was also accompanied by a reduction in the intracellular levels of GSH. A 28% reduction was achieved with the highest concentration of H_2O_2 used (9 μmoles/5×10^6 neutrophils). At a H_2O_2 concentration of 1 μmole in the preincubation mixtures there was no effect on zymosan-stimulated HMPS activity or the intracellular levels of GSH in H_2O_2-treated cells.

Hydrogen peroxide can itself stimulate the HMPS

Table 1. Hydrogen peroxide (H_2O_2) production, intracellular levels of reduced glutathione (GSH) and hexose monophosphate shunt (HMPS) activity in peroxide-treated and control neutrophils

	Control	Hydrogen peroxide-treated neutrophils 1 μmole	3 μmoles	6 μmoles	9 μmoles
H_2O_2 production (nmoles/0.25×10^5 cells)	7.6 ± 0.5	7.9 ± 0.5	8.4 ± 0.4†	9.2 ± 0.6‡	—
GSH content (nmoles/5×10^6 cells)	4.5 ± 0.45	4.45 ± 0.4	4.1 ± 0.5†	3.35 ± 0.45‡	3.25 ± 0.4‡
HMPS activity (nmoles CO_2 /2.5×10^6 cells)	7.3 ± 1.1	7.2 ± 0.9	6.6 ± 0.8	4.8 ± 0.4‡	4.4 ± 0.6‡

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$.

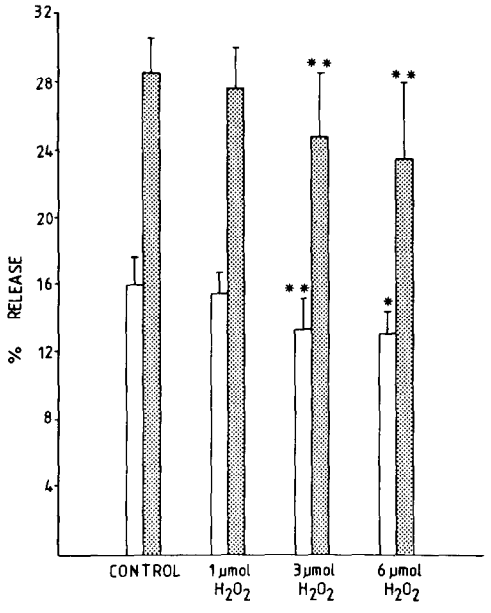

Fig. 4. Effect of H_2O_2-treatment of opsonized zymosan-stimulated release of lysosyme and myeloperoxidase (MPO) from neutrophils. □, lysozyme; ■, MPO. * $P < 0.02$; ** $P < 0.01$.

of glucose metabolism, in the absence of any additional stimulus. The H_2O_2 concentration which induces the optimal activation of the HMPS coincides with that which, when used in the preincubation mixtures with neutrophils, initiate the reduction in both GSH levels and HMPS activity of the cell (Fig. 5). This concentration of H_2O_2, 3 μmoles/5×10^6 neutrophils, was also that at which reduction in degranulation and early intracellular killing of *E. coli* were first detected.

Effect of SH-*containing compounds on the function of peroxide-treated neutrophils*

Phagocytosis and killing of E. coli. Preincubation with 3×10^{-4} M concentration of different SH-containing compounds resulted in increased *PI* values for *E. coli* in H_2O_2-treated neutrophils. The inhibition of early phagocytosis, induced by 3 μmoles of H_2O_2 (Fig. 6A), returned towards normal levels after treatment with all five compounds tested ($P < 0.02$ for GSH and $P < 0.01$ for other SH compounds). Phagocytosis by H_2O_2-treated cells was within the normal range in the later stages of incubation with *E. coli* and was not significantly different after treatment with SH-containing compounds. The killing of *E. coli* by H_2O_2-treated cells was similarly improved by preincubation with all SH-containing compounds ($P < 0.01$) except GSH, which was the only compound that had no effect in this assay system (Fig. 6C).

These compounds also had some beneficial effect on phatocytosis and killing of *F. coli* by neutrophils treated with 6 μmoles of H_2O_2 but the effects were considerably less pronounced (Fig. 6B, D).

O_2^- *and* H_2O_2 *production.* Superoxide production by H_2O_2-treated cells in response to opsonized zymosan was not greatly affected by preincubation with the SH compounds (Table 2). There was a significant elevation in the levels of O_2^- detected after preincubation with MMPC or α-MPG for neutrophils treated with either 3 or 6 μmoles of H_2O_2. The production of H_2O_2 by H_2O_2-treated cells remained elevated even after treatment with SH-containing compounds and, consequently, the H_2O_2/O_2^- molar ratio also remained elevated above normal values. A slight reduction in the H_2O_2/O_2^- ratio in neutrophils treated with 3 μmoles of H_2O_2 was caused by MMPC.

Release of granule enzymes. The release of lysozymes and myeloperoxidase from H_2O_2-treated neutrophils stimulated with opsonized zymosan was

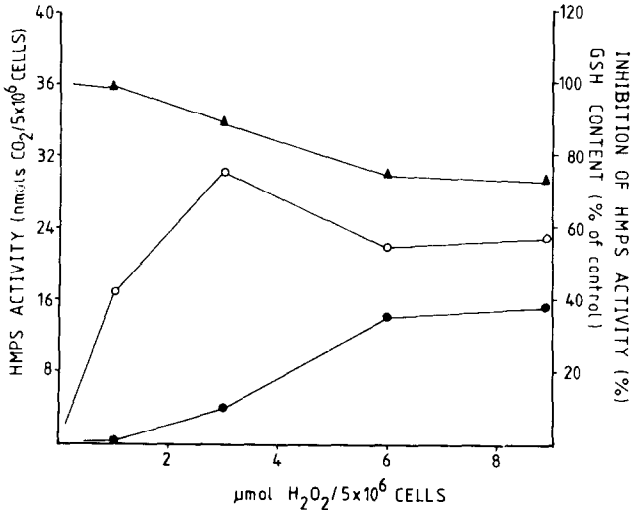

Fig. 5. Hydrogen peroxide-induced HMPS activity and the effects of H_2O_2-treatment on intracellular levels of GSH and opsonized zymosan-stimulated HMPS activity of neutrophils. ○, H_2O_2-induced HMPS activation; ▲, GSH content of cells; ●, % inhibition of zymosan-stimulated HMPS activity.

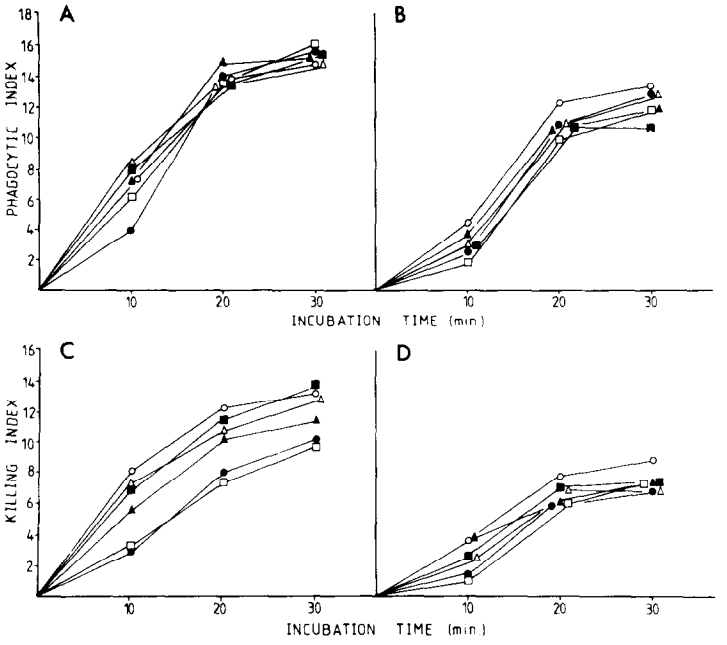

Fig. 6. Effect of SH-containing compounds on phagocytosis and killing of *E. coli* by neutrophils treated with either 3 μmoles (A and C) or 6 μmoles (B and D) of H_2O_2/5 × 10^6 cells. ●, H_2O_2-treated; ○, H_2O_2-treated + MMPC; □, H_2O_2-treated + GSH; ▲, H_2O_2-treated + PEN; △, H_2O_2-treated + α-MPG; ■, H_2O_2-treated + CYS.

Table 2. Effect of SH-containing compounds on H_2O_2-treated neutrophils: Superoxide (O_2^-)* and hydrogen peroxide (H_2O_2)† production in response to opsonized zymosan

	Control	GSH	MMPC	PEN	CYS	MPG
Neutrophils treated with:						
3 μmoles of H_2O_2						
O_2^- production	8.6 + 0.9	8.7 ± 0.7	9.1 ± 0.4‡	8.8 ± 0.7	8.7 ± 0.4	8.9 + 0.7§
H_2O_2 production	8.3 ± 0.6	8.4 + 1.0	8.2 + 0.4	8.3 + 0.8	8.1 ± 0.6	—
H_2O_2/O_2^-	0.97 ± 0.1	0.97 ± 0.09	0.90 ± 0.05	0.94 ± 0.08	0.93 ± 0.03	—
6 μmoles of H_2O_2						
O_2^- production	8.6 ± 0.9	8.7 ± 0.5	8.9 + 0.3§	8.6 ± 0.5	8.5 + 0.7	8.9 ± 0.4§
H_2O_2 production	9.3 ± 0.4	9.2 ± 0.2	9.1 ± 0.5	8.3 ± 0.3‡	8.8 ± 0.6§	—
H_2O_2/O_2^-	1.08 ± 0.05	1.06 ± 0.07	1.02 ± 0.09	0.97 + 0.06	1.04 + 0.09	—

* nmoles cytochrome c reduced/1.25 × 10^5 neutrophils.
† nmoles H_2O_2 produced/1.25 × 10^5 neutrophils.
‡ $P < 0.02$; § $P < 0.04$.

Table 3. Effect of SH-containing compounds on the opsonized zymosan-induced release (%) of granule enzymes from H_2O_2-treated neutrophils

	Control	GSH	MMPC	PEN	CYS	MPG
Neutrophils treated with:						
3 μmoles of H_2O_2						
Lysozyme	13.6 ± 2.6	13.5 ± 3.1	15.7 ± 1.8‡	15.9 ± 2.0‡	14.9 ± 0.9*	15.2 ± 1.5†
Myeloperoxidase	25.2 ± 3.0	25.4 ± 1.9	28.1 ± 2.2†	28.5 ± 3.5†	27.3 ± 0.8*	27.9 ± 1.9*
6 μmoles of H_2O_2						
Lysozyme	13.2 ± 1.8	13.0 ± 0.7	15.0 ± 1.6*	15.3 ± 2.4†	14.5 ± 0.9*	14.7 ± 0.8*
Myeloperoxidase	23.7 ± 2.7	23.2 ± 3.1	25.9 ± 2.3*	24.7 ± 1.9	26.3 ± 0.9*	25.1 ± 1.7

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$.

improved significantly after preincubation with four of the SH-containing compounds. Glutathione had no effect and the beneficial effect of the other compounds was considerably decreased in neutrophils treated with 6 μmoles of H_2O_2 (Table 3).

HMPS activity and intracellular levels of GSH. SH-containing compounds had very little effect on peroxide-induced inhibition of HMPS activity stimulated in neutrophils by opsonized zymosan. Even in neutrophils treated with 1 μmole of H_2O_2 there was no elevation in HMPS activity after treatment with SH compounds.

Similarly, GSH levels of H_2O_2-treated cells was not altered by preincubation with SH compounds for 60 min.

DISCUSSION

The assay for simultaneous estimation of phagocytosis and killing of bacteria employed in this study not only allowed confirmation of the earlier observation of reduced phagocytosis in H_2O_2-treated neutrophils [7] but also demonstration of the inhibitory effect of H_2O_2 on the killing of bacteria by the neutrophils. The first signs of H_2O_2-induced inhibition of phagocytosis were evident in the early stages of incubation in neutrophils preincubated with up to 3 μmoles H_2O_2. The killing process appeared to be more sensitive to inhibition by H_2O_2 in that even in neutrophils treated with 1 μmole of H_2O_2, defective killing of *E. coli* could be detected at the end of the incubation period. Although Boxer *et al.* [8] demonstrated C3b-receptor activity in H_2O_2-treated neutrophils, this is unlikely to be the mechanism of reduced phagocytosis in the present study since the opsonized zymosan-stimulated O_2^- production by H_2O_2-treated neutrophils remained normal at all times. Since O_2^- production is not dependent on the phagocytic process but only on the stimulus-receptor coupling, it would suggest that H_2O_2 primarily inhibits the ingestion phase and not the binding of the ligand to its receptor.

The H_2O_2-induced inhibition of bacterial killing by the neutrophils coincided with the inhibition of lysozyme release from the cells in response to opsonized zymosan. These results are in contrast to those of Anderson and Jones [16] who report increased spontaneous and stimulated release of alkaline phosphatase from neutrophils treated with H_2O_2 in combination with horseradish peroxidase and iodine. In the present study the reduced degranulation was accompanied by depletion of intracellular levels of GSH. The H_2O_2 within the peroxidase/halide complex may not be accessible to the glutathione redox system which may explain the difference in results obtained with reagent H_2O_2 and its complexed form. Glutathione-oxidizing agents, such as diamide or *t*-butyl hydroperoxide, promote concanavalin A-receptor capping and inhibit microtubule assembly as demonstrated by inhibitory effects of anti-tubulin drug, colchicine, on degranulation [18]. The inhibitory effect on degranulation of neutrophils may then be related to its ability to deplete the intracellular stores of GSH. The role of GSH in the detoxification of H_2O_2 are further supported by our findings of increased H_2O_2/O_2 molar ratio in stimulated neutrophils previously treated with peroxide. Catalase also plays a role in removal of excess peroxide but this enzyme has a low affinity for H_2O_2 and cannot cope well with low concentrations of this oxygen metabolite. The possibility that catalase may be localized in the peroxisomes [19] would also make it less available to H_2O_2 present in the extracellular or cytoplasmic compartments. In contrast to catalase, glutathione redox system has a very high affinity for H_2O_2 and the components of the system are found with the cytoplasm of most mammalian cells, easily accessible to low concentrations of H_2O_2 whether generated intra, or extracellularly [5].

The concentration of H_2O_2 at which the reduction of cellular GSH was first detectable coincided with that which caused depressed degranulation, HMPS activity and intracellular killing of *E. coli*. This would suggest a close relationship between neutrophil function and intracellular levels of GSH. Studies of neutrophil locomotion have shown that SH-containing compounds such as cysteine or α-MPG can potentiate this neutrophil function and that this effect may be the result of their antioxidant properties [16, 20, 21]. We have shown previously [10] that SH-containing compounds, when present in the reaction mixtures with neutrophils, can reduce the amount of O_2^- and H_2O_2 detected in the extracellular environment. This is not due to interference with oxygen-metabolite production by the cells since HMPS activity is increased in their presence and the intracellular killing of *E. coli* by the neutrophils remained normal. The metabolic activity of neutrophils, which is self-limiting, may be enhanced by removal of the produced oxygen metabolites thus protecting the cells from their toxic effect.

The present study demonstrates that the SH-containing compounds are able to reverse the H_2O_2-induced inhibition of neutrophil function. An earlier study by Anderson and Jones [16] has already shown that cysteine can reverse the toxic effects of horseradish peroxidase/H_2O_2/iodide system on neutrophil locomotion. Most thiols used in the present study were able to improve phagocytosis of bacteria by H_2O_2-treated cells. The improvement in the killing capacity of the neutrophils was accompanied by increased degranulation despite the reduced levels of GSH, which remained reduced even after treatment with SH-containing compounds. Glutathone, used extracellularly, was not effective in improving either the killing of *E. coli* or degranulation which would be consistent with its inability to permeate the plasma membrane of intact cells. The beneficial effects of other compounds in this system would then suggest that they may be able to cross the membrane and exert their effects intracellularly. This still remains to be established.

How much H_2O_2 is released extracellularly by stimulated neutrophils *in vivo* is uncertain but it is possible that very high concentrations could build up at the plasma membrane surface or in the small spaces within the neutrophil aggregates. The ability of SH-containing compounds to protect the neutrophil from, and reverse the toxic effects of, oxygen metabolites such as H_2O_2 may have particular relevance in the therapy of chronic autoimmune and inflammatory diseases in which reduced neutrophil

responses may be caused by auto-oxidative injury and lead to increased susceptibility to bacterial infection.

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