Neutrophil degranulation inhibits potential hydroxyl-radical formation

Relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques

Bradley E. BRITIGAN,* Daniel J. HASSETT,‡‡ Gerald M. ROSEN,§§ Danielle R. HAMILL* and Myron S. COHEN††

*Department of Medicine, Veterans Administration Medical Center and The University of Iowa College of Medicine, Iowa City, IA 52242, Departments of ‡Medical and ††Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599, §Department of Pharmacology and Toxicology, University of Maryland School of Pharmacy, Baltimore, MD 21201, and ‡†Veterans Administration Medical Center, Baltimore, MD 21218, U.S.A.

INTRODUCTION

The microbicidal and/or inflammatory activity of human neutrophils is linked to their ability to form superoxide anion (O$_2^-$) and H$_2$O$_2$ [2]. H$_2$O$_2$ and O$_2^-$ react in vitro in the presence of iron to form hydroxyl radical (·OH) via the Haber–Weiss reaction [3,4]. In the absence of iron (or another transition metal) the reaction rate is too low to have biological relevance. ·OH has been suggested to contribute to both the microbicidal activity and the tissue damage associated with neutrophil activation in vivo [5]. Several investigations have reported detection of ·OH formed by stimulated neutrophils suspended in vitro in standard buffers [6–15]. These studies implied that neutrophils possessed the endogenous capacity for ·OH formation. However, the specificity of the experimental techniques and ‘scavenger agents’ used to assess neutrophil ·OH detection in these studies has been questioned [16–24]. In addition, the possibility was not excluded that exogenous iron contaminating the buffer systems allowed ·OH to be produced.

Recent work in several laboratories has forced re-evaluation of original assumptions regarding the endogenous capacity of neutrophils to generate ·OH [25–34]. Studies employing m.s. [32,33], deoxyribose oxidation ([30,31]; B. E. Britigan, unpublished work), phenylalanine hydroxylation [34], and spin trapping [25–29] have failed to detect production of ·OH by neutrophils unless iron salts and a chelating agent were included in the system. These studies suggest that generation of ·OH in vitro in association with neutrophil stimulation requires the presence of an exogenous catalyst.

Even in the presence of an exogenous iron catalyst, we previously presented ‘in vitro’ evidence, using spin-trapping techniques, that release of granule lactoferrin (LF) from stimulated neutrophils inhibited ·OH production [27], presumably by binding iron in a non-catalytic form [35–38]. Recently the possibility was raised [39] that neutrophil-mediated destruction of spin-trapped adducts used to monitor ·OH formation could have confounded interpretation of some of these data. In contrast with our [27] results, Winterbourn [30] reported

Abbreviations used: ·OH, hydroxyl radical; MPO, myeloperoxidase; O$_2^-$, superoxide anion; LF, lactoferrin; anti-LF, polyclonal rabbit anti-(human LF) antibody; DTPA, diethylenetriaminepenta-acetic acid; SOD, superoxide dismutase; FMLP, N-formylmethionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; Me$_3$SO, dimethyl sulphoxide; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; HBM, Hanks balanced salt solution without Phenol Red; DMPO-OOH, 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxy; DMPO-OH, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxy; Ch$_3$, methyl radical; DMPO-CH$_3$, 2,2,5-trimethyl-1-pyrrolidinyloxy; N$_3^-$, azide (NaN$_3$).
that myeloperoxidase (MPO) released during neutrophil stimulation could inhibit the magnitude of \( \cdot \text{OH} \) formed (thiobarbituric acid-reactive 2-deoxyribose oxidation products) by the addition of iron-EDTA to a xanthine/xanthen oxide \( \text{O}_2^- \)-generating system. This presumably occurred as a consequence of MPO removing \( \text{H}_2\text{O}_2 \) and/or \( \text{O}_2^- \) from the system [40,41]. Although these data suggested release of MPO would also inhibit formation of \( \cdot \text{OH} \) by iron-supplemented neutrophils, this hypothesis was not directly tested. Methodological differences between the studies of Winterbourn [30] and our own [27] precluded their direct comparison. Consequently, the present work was undertaken to determine the relative impact of MPO and LF release on the potential for neutrophil \( \cdot \text{OH} \) formation.

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**MATERIALS AND METHODS**

Reagents

Diethylenetriaminepenta-acetic acid (DTPA), cytochalasin B, ferricytochrome c, hypoxanthine, xanthine oxidase, superoxide dismutase (SOD), catalase, \( \text{N} \)-formyl-methionyl-leucylphenylalanine (FMLP) and zymosan were purchased from Sigma Chemical, St. Louis, MO, U.S.A. Phorbol myristate acetate (PMA) was obtained from Midland Chemical Co., Brewster, NY, U.S.A., and dimethyl sulfoxide (Me\(_2\)SO) and NaN\(_3\), from Fisher Scientific, Fair Lawn, NJ, U.S.A. 5,5-Dimethyl-1-pyrroline 1-oxide (DMPO) was synthesized by the method of Bonnett et al. [42] or purchased from Sigma and used without further purification. Zymosan was opsonized by incubation in 100% normal pooled human serum (six to eight healthy donors) for 30 min, followed by three washes in normal saline to a concentration of 100,000 colony-forming units (cfu)/ml in Hanks balanced salt solution without Phenol Red (HBSS) on ice until use. Polyclonal rabbit anti-human lactoferrin (anti-LF) was purchased from Cappel Laboratories, Cochranville, PA., U.S.A.

Neutrophil separation

Blood was drawn from healthy volunteers into heparinized syringes. Plasmagel (Roger Bellon, Neuilleu, France [43]) or dextran sedimentation [44] was employed to separate leukocytes and erythrocytes. Experimental results were independent of the method of cell separation. The leukocyte-containing fraction was removed and sedimented through a Ficoll/Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). The neutrophil pellet was washed, and contaminating erythrocytes were removed by osmotic lysis. Neutrophils were suspended in HBSS and concentrations were adjusted by using a model D2N automated blood-cell counter (Coulter Electronics, Hialeah, FL, U.S.A.). In experiments employing neutrophils pretreated with cytochalasin B, cells were incubated in the presence of cytochalasin B (5 \( \mu \text{g/ml} \)) for 10 min before use. In some experiments neutrophils were obtained from an individual whose cells were totally deficient in MPO as determined by both the \( \text{o} \)-dianisidine MPO assay and Western-blot analysis [45].

**O\(_2\) consumption**

\( \text{O}_2 \) consumption was measured in a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Neutrophils [(0.5–1.5) \( \times 10^7 \text{/ml} \)] were incubated in 1 ml of HBSS at 37 °C. After the addition of the desired stimulus, \( \text{O}_2 \) saturation was recorded continuously and results were expressed as maximal \( \text{O}_2 \) consumption rate (nmol/min) observed [43]. Azide (N\(_3^-\); 1–10 mm) increased PMA- and opsonized-zymosan-stimulated \( \text{O}_2 \) consumption by 20.9 \( \pm \) 7.3% (\( n = 6 \)) and 26.4 \( \pm \) 8.7% (\( n = 5 \)) respectively. Neither of these increases was statistically significant (\( P > 0.05 \)) by Student’s paired \( t \) test.

**Superoxide release**

Neutrophil superoxide release was measured as the SOD-inhibitable reduction of ferricytochrome \( c \), as previously described [46]. Neutrophils [(1–20) \( \times 10^6 \text{/ml} \)] were placed in 1 ml of HBSS containing ferricytochrome \( c \) (80 \( \mu \text{m} \)), with or without cytochalasin B (5 \( \mu \text{g/ml} \)), in the reference cuvette of a Perkin–Elmer model 57 spectrophotometer (Perkin–Elmer, Mountain View, CA, U.S.A.). After the addition of PMA (100 ng/ml), opsonized zymosan (3 mg/ml) or FMLP (10\(^{-8}\) M), absorbance was recorded continuously at 550 nm and 25 °C. DMPO did not affect ferricytochrome \( c \) reduction by \( \text{O}_2^- \) generated with a xanthine oxidase/hypoxanthine system.

**Spin trapping**

Spin trapping was performed as previously described [25–29]. Immediately after the addition of the desired stimulus, reaction mixtures (0.5 ml final vol.) containing neutrophils [(0.5–2.0) \( \times 10^7 \text{/ml} \), DMPO (0.1 m), Me\(_2\)SO (0.14 m or 0.28 m), DTPA (0.1 m), with or without iron (0.1 m)] were transferred to a flat quartz cell and fitted into the cavity of the e.p.r. spectrometer (model E-9 or E104A; Varian Associates, Palo Alto, CA, U.S.A.). Iron utilized in these experiments consisted of ferrous ammonium sulphate that had been added to phosphate buffer, pH 7.4. Under these conditions ferrous iron is rapidly oxidized to yield a solution of nearly 100% ferric iron [47]. E.p.r. spectra were recorded at 25 °C in sequential 6 min scans. Unless noted otherwise, e.p.r. spectra were recorded with a microwave power of 20 mW, modulation frequency of 100 kHz with an amplitude of 0.1 mT, sweep time of 1.25 mT/min, and receiver gain of 3.2 \( \times 10^4 \) with a response time of 1 s. Neutrophil stimuli employed were PMA (100 ng/ml), opsonized zymosan (3 mg/ml) or FMLP (10\(^{-8}\) M). In some experiments reaction mixtures also contained SOD (30 units/ml), catalase (3–500 units/ml), azide (1–10 mM), anti-LF (10–100 \( \mu \text{g} \)/ml) and cytochalasin B (5 \( \mu \text{g/ml} \)) alone or in combination. One unit of catalase is defined as that amount of the enzyme which will decompose 1.0 \( \mu \text{mol} \) of \( \text{H}_2\text{O}_2 \)/min at pH 7.0 and 25 °C. SOD activity was determined by the method of McCord & Fridovich [48]. The optimal anti-LF concentration for the observation described varied from lot to lot. The final Me\(_2\)SO concentration was always maintained at either 0.14 m or 0.28 m for each set of experiments. In some cases, experiments were conducted in which a superoxide-generating system consisting of hypoxanthine (0.5 mM) in the presence of xanthine oxidase (0.1 unit/ml) was substituted for neutrophils.
Statistical analysis

Paired or unpaired Student’s t tests were used for statistical analysis, with results considered significant at $P < 0.05$. Although data are sometimes expressed as percentage of appropriate control, raw data were used for statistical purposes.

RESULTS

Inhibition of the iron-catalysed formation of ‘OH by PMA-stimulated neutrophils

The spin trap DMPO reacts with $\mathcal{O}_2^-$ and ‘OH to yield the spin-trapped adducts 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinylloxyl (DMPO-OOH) and 2,2-dimethyl-5-hydroxyl-1-pyrrolidinylloxyl (DMPO-OH) respectively [19]. However, DMPO-OOH decomposes to DMPO-OH, ‘OH and a diamagnetic species [19], making detection of DMPO-OH unreliable as evidence for ‘OH. In the presence of Me$_3$SO, ‘OH leads to the formation of methyl radical (‘CH$_3$), which can be spin-trapped with DMPO as 2,2,5-trimethyl-1-pyrrolidin voxyl (DMPO-CH$_3$) [19,25-29]. The reaction kinetics of ‘OH with DMPO and Me$_3$SO are similar. Therefore when experimental conditions are created in which the concentration of Me$_3$SO exceeds DMPO, ‘OH production is manifested primarily as DMPO-CH$_3$ [19; 25-29]. Since DMPO-CH$_3$ appears to be formed only in the presence of ‘OH, this provides a more specific ‘OH detection system than that employing DMPO alone. It should be noted that $\mathcal{O}_2^-$ generation alone will result in the formation of a small amount of DMPO-CH$_3$ as a consequence of direct DMPO-OOH decomposition to ‘OH and its resulting interaction with Me$_3$SO to yield ‘CH$_3$ [25,26]. DMPO-CH$_3$ formed via this mechanism is easily differentiated from that resulting from Haber–Weiss-mediated ‘OH generation, since catalase inhibits Haber–Weiss-mediated DMPO-CH$_3$ formation, but not that resulting from DMPO-OOH decomposition.

When neutrophils were stimulated with PMA in the presence of DMPO, Me$_3$SO, iron, and DTPA, DMPO-CH$_3$ (catalase-inhibitable) initially was the dominant spin-trapped adduct (Fig. 1). However, with sequential scans, DMPO-CH$_3$ peaks decreased, whereas DMPO-OOH continued to accumulate (Fig. 1), reflected as a progressive decrease in the ratio of DMPO-CH$_3$/DMPO-OOH peak amplitudes (Fig. 2). These spectra are in marked contrast with what is observed with the addition of the same concentration of iron to a hypoxanthine/xanthine oxidase $\mathcal{O}_2^-$-generating system, where DMPO-CH$_3$ remains dominant over sequential scans [26-28]. Although other possible mechanisms could explain this observation, and were examined (see below), it seemed most likely that a decrease in the rate of ‘OH formation occurred over time. The continued accumulation of $\mathcal{O}_2^-$-derived spin-trapped adducts suggested that decreasing $\mathcal{O}_2^-$ generation was not responsible.

The impact of neutrophil MPO and LF release on the above process was assessed by repeating experiments in the presence of NaN$_3$ (to inhibit MPO) or anti-LF. NaN$_3$ increased the DMPO-CH$_3$ peak amplitude observed during the first 6 min of the experiment by 50–100%, but did not prolong the duration of apparent ‘OH production (Fig. 2). Anti-LF also increased initial DMPO-CH$_3$ peak amplitudes. In addition, it markedly prolonged the duration of apparent ‘OH production, as reflected by the sustained DMPO-CH$_3$/DMPO-OOH ratio of greater than 1 for more than 20 min (Fig. 2). The simultaneous presence of NaN$_3$ and anti-LF yielded scans identical with that obtained with anti-LF alone (results not shown).

NaN$_3$ also inhibits catalase activity [49]. Given the importance of the concentration of $\mathcal{H}_2\mathcal{O}_2$ to the Haber–Weiss reaction, experiments were performed to eliminate the possibility that inhibition of neutrophil catalase was responsible for the effect of NaN$_3$ observed. Neutrophils which were totally MPO-deficient were stimulated with PMA in the presence of iron, and the effect of NaN$_3$ was assessed. As shown in Fig. 3, NaN$_3$ did not enhance DMPO-CH$_3$ observed with MPO-deficient neutrophils.

Stimulation with opsonized zymosan

Neutrophil stimulation with PMA reportedly results in preferential specific-granule release [50]. Because speci-
Fic granules are rich in LF and do not contain MPO [51], results with PMA could underestimate the impact of MPO on 'OH formation by iron-supplemented neutrophils. Therefore experiments were repeated with opsonized zymosan, which evokes secretion of both primary (MPO-rich) and secondary granule controls. Neutrophils exposed to opsonized zymosan in the presence of iron yielded e.p.r. spectra similar to those obtained with PMA (Fig. 4). As with PMA, DMPO-CH$_3$ was the dominant species in the first scan, but with time DMPO-CH$_3$ decreased and DMPO-OOH became the dominant species (Figs. 4 and 5). With opsonized zymosan an increase in the relative magnitude of DMPO-OOH was also observed (Fig. 4). We and others [25, 26, 28, 39] have previously shown that neutrophil stimulation induced by opsonized zymosan leads to greater detection of DMPO-OOH by a mechanism that remains a subject of controversy [28, 39]. Since it was impossible to determine what proportion of DMPO-OOH detected resulted from O$_2^\cdot$ as compared with 'OH production, DMPO-OOH peak amplitudes were not considered when calculating ratios of 'OH- to O$_2^\cdot$-derived spin-trapped adducts (DMPO-CH$_3$/DMPO-OOH ratios). The results presented for opsonized zymosan stimulation therefore probably underestimate the rate of conversion into a spectrum dominated by O$_2^\cdot$-derived spin-trapped adducts ('O$_2^\cdot$-dominated').

In the presence of N$_3^\cdot$, DMPO-CH$_3$ peak amplitudes doubled after neutrophil stimulation with opsonized zymosan (Fig. 5). Similarly to results with PMA, anti-LF doubled initial DMPO-CH$_3$ peaks in response to opsonized zymosan and slowed the progression to a 'O$_2^\cdot$-dominated' spectrum (Fig. 5). To try to match the experimental conditions used by Winterbourn [30], we attempted to examine formation of 'OH by FMLP-stimulated neutrophils with the above-mentioned spin-trapping system. However, this proved to be impossible, as 0.1 mM-DMPO inhibited FMLP-stimulated neutrophil with the above-mentioned spin-trapping system. This appears to result from the high sensitivity of DMPO to inhibit FMLP-induced neutrophil stimulus-response coupling (B. E. Britigan & D. R. Hamill, unpublished work). Furthermore, Fe-EDTA, the iron chelate employed by Winterbourn [30], cannot be reliably used with DMPO, since its presence results in significant oxidation of DMPO to stable nitroxides [19].

**Effect of N$_3^\cdot$ and anti-LF on 'OH formation by cytochalasin B-pretreated neutrophils**

The phagocytic vacuole formed in response to stimuli such as opsonized zymosan (but not PMA) creates a...
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Fig. 4. E.p.r. spectra of opsonized-zymosan stimulation of neutrophils suspended in the presence of exogenous iron

Three sequential (6 min/scan) e.p.r. spectra obtained immediately after the addition of opsonized zymosan (3 mg/ml) to neutrophils (10⁷/ml) suspended in HBSS containing Me₃SO, DMPO, DTPA and iron (0.1 mM) are shown. High- and low-field peaks corresponding to DMPO-CH₃, DMPO-OH, and DMPO-OH are designated as in Fig. 1. As seen with PMA (Figs. 1 and 2), the e.p.r. spectrum initially dominated by DMPO-CH₃ evolved to an O₂⁻-derived-adduct dominated spectrum (DMPO-OH/OOH) over time. Spectra are representative of five experiments.

Fig. 5. Effect of N₃⁻ and anti-LF on the magnitude and duration of 'OH production after opsonized-zymosan stimulation of iron-supplemented neutrophils

Ratios of high- and low-field peaks of DMPO-CH₃ and DMPO-OOH observed over time after opsonized-zymosan stimulation of neutrophils (10⁷/ml) suspended in HBSS containing Me₃SO, DMPO, DTPA and iron (●), as well as 1 mM-N₃⁻ (△) or anti-LF (10 µg/ml; ○) are shown. N₃⁻ enhanced the magnitude, but not the duration, of apparent 'OH production, whereas anti-LF increased both. Results are representative of four separate experiments.

Fig. 6. Effect of N₃⁻ and anti-LF on 'OH production after opsonized-zymosan stimulation of neutrophils pretreated with cytochalasin B in the presence of exogenous iron

Ratios of high- and low-field DMPO-CH₃ and DMPO-OOH peaks observed in sequential e.p.r. spectra obtained after opsonized-zymosan addition to cytochalasin B-treated neutrophils suspended in the presence of Me₃SO, DMPO, DTPA and iron (●) are shown. Addition of 10 mM-N₃⁻ (△) increased the magnitude of 'OH production (initial increase in DMPO-CH₃/DMPO-OOH ratio), but not its duration of production. Addition of anti-LF (10 µg/ml) increased both (○). Results are representative of three separate experiments.

milieu not necessarily accessible to compounds used to detect free-radical formation or scavenging agents used to block their actions. Available data suggest that DMPO is capable of penetrating vacuolar sites sufficiently to detect phagosomal free-radical formation [25,26,52]. However, by restricting access of N₃⁻ and/or anti-LF, the phagosome might limit one’s ability to measure accurately the effects of LF and MPO at this critical site. Therefore experiments were repeated with neutrophils treated with cytochalasin B, which inhibits phagosome closure [2]. Consistent with our earlier observations [25,26], in response to opsonized zymosan cytochalasin B pretreatment decreased spin-trapped-adduct peak amplitudes by approx. 50%, a phenomenon presumably attributable to cytochalasin B-mediated inhibition of neutrophil O₂⁻ formation [25]. In addition, cytochalasin B decreased the rate of decrease of the DMPO-CH₃/DMPO-OH peak ratios. This was likely due to the lower rate of O₂⁻ production, which resulted in a slower accumulation of DMPO-OH and possibly increased DMPO-CH₃ stability ([39]; see below). As observed above (Fig. 5), N₃⁻ increased the magnitude, but not the duration, of apparent 'OH formation, whereas anti-LF increased both (Fig. 6).

Spin-trapped-adduct stability and data interpretation

Neutrophil stimulation converts a variety of nitroxides, including DMPO-OH and DMPO-CH₃, to diamagnetic
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Fig. 7. Effect of SOD on EPR spectra following stimulation of iron-supplemented neutrophils

(a) Two sequential e.p.r. spectra recorded beginning immediately after the addition of SOD (30 units/ml) to neutrophils (5 x 10⁶/ml) suspended in the presence of Me₂SO, DTPA, DMPO and iron, which had been stimulated with PMA 7 min earlier; (b) same as (a), except that catalase (300 units/ml) was added along with SOD 7 min after the addition of PMA. Catalase prevented the accumulation of DMPO-CH₃ peaks observed in the presence of SOD. High- and low-field DMPO-CH₃, DMPO-OH and DMPO-OOH peaks designated as in Fig. 1. Results representative of three separate experiments. The receiver gain was 3.2 x 10⁴.

species that are, therefore, not detectable by e.p.r. [28,29,39]. Since the present studies were conducted in the presence of exogenous iron and resulted in initial e.p.r. spectra consistent with 'OH spin trapping, the capacity of the system to detect 'OH under these conditions is not an issue. Nevertheless, it seemed possible that the effect of N₃⁻ and/or anti-LF described above could be related to increased DMPO-CH₃ stability rather than increased 'OH production. Several experimental approaches were taken to examine the possibility. The decrease in DMPO-CH₃ peak amplitudes noted 5–10 min after neutrophil stimulation indicated a much shorter half-life for this adduct than that observed in cell-free systems [19]. Previous work [28,29,39] made it likely that the presence of neutrophil-derived O₂⁻, in conjunction with a thiol-like compound, was responsible. Consistent with this hypothesis, addition of SOD 7 min after stimulation of iron-supplemented neutrophils increased DMPO-CH₃ peaks observed over sequential scans (Fig. 7). However, at least part of the SOD effect likely involved accelerated formation of 'OH. Analogous to the more detailed assessment of this phenomenon [29], addition of SOD and catalase 7 min after stimulation of iron-supplemented neutrophils yielded e.p.r. spectra with only small relatively stable DMPO-CH₃ peaks (Fig. 7). Addition of anti-LF to stimulated neutrophils had no effect on the rate of neutrophil O₂⁻ production (O₂ consumption, ferricytochrome c reduction), nor did anti-LF exhibit SOD-like activity (measured by its ability to inhibit hypoxanthine/xanthine oxidase-mediated ferricytochrome c reduction). Anti-LF had no effect on peak amplitudes resulting from stimulation of neutrophils suspended in the absence of exogenous iron (results not shown). Thus the ability of anti-LF to increase DMPO-CH₃ peaks resulting from stimulation of iron-supplemented neutrophils could not be explained on the basis of increased DMPO-CH₃ stability.

MPO-derived oxidants may convert both DMPO-OH and DMPO-CH₃ into diamagnetic species [53,53a]. However, given the high rate of DMPO-OH and DMPO-CH₃ decomposition occurring as a consequence of neutrophil stimulation [29,39], the relative impact of MPO on spin-trapped-adduct stability was unclear. Small DMPO-CH₃ peaks are observed when neutrophils are stimulated in the absence of iron, owing to DMPO-OOH decomposition to 'OH and its subsequent reaction with Me₂SO to form CH₃. Consequently, e.p.r. spectra were obtained after PMA or opsonized-zymosan stimulation of neutrophils suspended in HBSS containing DMPO, Me₂SO and DTPA in the absence or presence of N₃⁻. N₃⁻ had no effect on the peak heights of DMPO-OH or DMPO-CH₃, but increased DMPO-OOH (results not shown), consistent with the known ability of N₃⁻ to increase neutrophil O₂⁻ production [54,55]. Also, consistent with a lack of significant effect of MPO on DMPO-CH₃ stability, the rate of DMPO-CH₃ peak amplitude decrease observed after stimulation of iron-supplemented neutrophils in the presence of azide was similar to the control value (Figs. 2 and 4).

DISCUSSION

The rate and magnitude of 'OH formation via the Haber–Weiss reaction are affected by the concentrations of O₂⁻ and H₂O₂, the availability of transition-metal catalysts such as iron, and possibly the ratio of Fe²⁺ to Fe³⁺ [56], although this remains controversial [57]. The present study was conducted to examine the relative impact of MPO and LF release on 'OH formed by human neutrophils provided with exogenous iron in a form capable of catalysing the Haber–Weiss reaction. Spin trapping was employed because this procedure allows simultaneous 'on-line' discrimination between 'OH and O₂⁻, as well as an insight into the magnitude and duration of free-radical production. Furthermore, available evidence suggests that spin trapping detects phagosomal events [25,26,52].

In the present work, experimental conditions were created such as to be sure that 'OH generation occurred as a consequence of neutrophil O₂⁻ reduction. In the presence of iron and DTPA, spin-trapped adducts consistent with neutrophil 'OH generation were seen with
both PMA and opsonized zymosan, yielding c.p.r. spectra which were initially dominated by DMPO-CH$_3$. However, over time, DMPO-CH$_3$ peak amplitude declined, whereas spin-trapped adducts resulting from O$_2^-$ production (DMPO-OOH and DMPO-OH) continued to accumulate. These data suggested a decrease in the rate of \('OH\) formation. As previously noted [27], the continued accumulation of O$_2^-$-derived spin-trapped adducts eliminated cessation of O$_2^-$/H$_2$O$_2$ formation as an explanation, thereby suggesting that a decrease in available catalyst was responsible.

N$_3^-$ inhibits the activity of haem-containing enzymes such as MPO. N$_3^-$ doubled the magnitude of DMPO-CH$_3$ formation observed after stimulation of iron-supplemented neutrophils without affecting the duration of its detection. Although it has been shown that N$_3^-$ can act as an ‘OH scavenger [58] at the concentration employed, it had no effect on the spin trapping of ‘OH formed by an iron-supplemented enzymic O$_2^-$-generating system. As with previous studies [54,55], we noted that N$_3^-$ induced a 20–30% increase in neutrophil O$_2^-$ consumption (and therefore O$_2^-$ generation) in response to PMA or opsonized zymosan. Since production of a single molecule of ‘OH via the Haber–Weiss reaction requires three molecules of O$_2^-$, such a small increase in O$_2^-$ formation could not account for the 2-fold increase in ‘OH production seen with N$_3^-$ Control experiments with MPO-deficient neutrophils further eliminated this possibility, as well as the potential contribution of N$_3^-$-mediated inhibition of neutrophil catalase to the process.

Our results, which are consistent with the recent work of Winterbourn [30], stand in sharp contrast with earlier reports in which detection of oxidation products of methional, 2-oxo-4-thiomyethylbutyric acid [6,7,16], benzoic acid [9–11], Me$_2$SO [8] and salicylate [12] were offered as evidence for the endogenous capacity of neutrophils to produce ‘OH. In these studies, N$_3^-$ uniformly inhibited oxidation of these substrates by neutrophils suspended in standard buffers. The authors of these reports generally chose to interpret their results as evidence that neutrophils formed ‘OH by an MPO-dependent mechanism other than the Haber–Weiss reaction. More recent data [16–24] indicate that these assays are relatively non-specific and may yield similar oxidation products as a consequence of the presence of an MPO-derived oxidizing agent other than ‘OH.

In contrast with the above results, anti-LF increased both the magnitude and duration of DMPO-CH$_3$ detection after stimulation of iron-supplemented neutrophils with opsonized zymosan or PMA. Anti-LF had no significant effect on DMPO-CH$_3$ resulting from stimulation of non-iron supplemented neutrophils or, as we previously demonstrated [27], by iron-supplemented neutrophilic HL-60 cells (which are deficient in LF).

The relative importance of granular proteins to neutrophil-associated ‘OH formation has previously been shown by demonstrating that cell-free supernatant obtained after neutrophil stimulation inhibited enzymically generated ‘OH [27,30]. In the work of Winterbourn [30], almost all of this inhibition could be prevented by inhibiting MPO activity, whereas we previously demonstrated a role for LF in the process [27]. These differences are likely explained by variations in experimental conditions employed, such as neutrophil concentration, stimulus and exogenous iron chelate. Of particular note, LF is not able to remove iron from EDTA [59], which was the chelate employed by Winterbourn [30]. In contrast with our data, some earlier reports suggested that LF could catalyse ‘OH generation by stimulated neutrophils [60,61]. This hypothesis has been questioned extensively [35–38], and it seems unlikely that the conditions required for potential ‘OH catalysis by this protein occur in vivo [37].

LF is predominantly secreted extraphagosomally, whereas MPO is deposited mostly within the phagosome [50], suggesting the two compounds might have relatively different effects on intra- and extra-phagosomal ‘OH production. Cytochalasin B-treated cells were examined to determine to what extent N$_3^-$ and anti-LF interfered with intraphagosomal events. The rate of DMPO-CH$_3$/DMPO-OOH decrease was lower with cytochalasin B. This probably relates to the lower rate of O$_2^-$ formation we have previously noted under such conditions [25], which could result in a lower rate of DMPO-OOH accumulation and/or increased DMPO-CH$_3$ stability. Cytochalasin B-treated neutrophils also exhibited an increase in the magnitude, but not the duration, of ‘OH generation in the presence of N$_3^-$ This is consistent with the results of earlier studies which demonstrated the ability of N$_3^-$ to inhibit neutrophil protein iodination and imply that N$_3^-$ penetrates the phagosome [58]. In the absence of anti-LF, the DMPO-CH$_3$/DMPO-OOH ratio exhibited a lower rate of decrease with cytochalasin B relative to normal neutrophils. However, given the lower rate of DMPO-CH$_3$/DMPO-OOH ratio decrease observed with cytochalasin B-treated neutrophils in the absence of anti-LF, cytochalasin B probably had little effect on the impact of anti-LF. This would be predicted, since LF is predominantly secreted extracellularly [50].

Recent work has demonstrated that the stability of preformed DMPO spin-trapped adducts is decreased in neutrophil-containing systems [28,39]. Destruction of DMPO-CH$_3$ associated with neutrophil stimulation likely played a role in the decreased stability of DMPO-CH$_3$ observed in the present study. However, this by itself would not account for the sequential c.p.r. spectra we observed for a number of reasons. First, the rate of neutrophil O$_2^-$ generation is highest in the first few minutes after stimulus addition, and then reaches a relatively steady level [62]. Consequently, one would expect delayed, followed by stable, DMPO-CH$_3$ peaks if destruction of the adduct mediated by events associated with the neutrophil respiratory burst was the sole event occurring. Second, whereas DMPO-CH$_3$ decreased over time, a continued accumulation of DMPO-OH, which appears to be as susceptible as DMPO-CH$_3$ to neutrophil-mediated destruction [29,39], was noted. Finally, a stable DMPO-CH$_3$ spectrum has been observed [27,63] when other phagocytes and myeloid cell lines are stimulated in the presence of iron at concentrations which result in rates of O$_2^-$ generation associated with neutrophil DMPO-CH$_3$ destruction [28,39]. Additional control experiments performed in the present study demonstrated that neither N$_3^-$ nor anti-LF effects could be explained on the basis of increased DMPO-CH$_3$ stability rather than ‘OH formation. Recent work [29] suggests that experiments assessing the stability of preformed DMPO spin-trapped adducts likely overestimate the importance of spin-trapped-adduct destruction in biological systems in which a continuous flux of ‘OH is expected.

Our results therefore suggest that both neutrophil MPO and LF release can inhibit formation of ‘OH
in vitro by iron-supplemented neutrophils. MPO presumably acts by consumption of H$_2$O$_2$ and O$_2^-$, and LF by binding iron in a non-catalytic form. The relative impact of MPO and lactoferrin on the production of 'OH by neutrophils in vivo is not clear. Hereditary MPO deficiency is not associated with severe inflammatory processes [64]. Intraplagosomal MPO may not have any impact on extraplagosomal 'OH production. In fact, if microbial iron is capable of catalysing the Haber–Weiss reaction [65–67], intraplagosomal 'OH production might help to explain the near-normal microbial activity of MPO-deficient neutrophils [64]. Studies in vitro of neutrophils obtained from the small number of patients with specific-granule (LF) deficiency have revealed multiple abnormalities [68]. The role of LF in the recurrent infections noted in those patients is unclear. The lack of severe inflammatory tissue damage observed may attest to the effectiveness of other iron chelators present in vivo.

In summary, available data suggest that production of 'OH in vivo as a consequence of neutrophil O$_2$ reduction requires unique environmental conditions which: (1) provide the phagocyte with an 'OH catalyst and (2) overwhelm or by-pass the ability of MPO and LF to inhibit 'OH generation. Appropriately, attention is now being focused on the potential for neutrophil targets or other components of the microenvironment to be a source of catalytic iron [65–67,69–73]. It is clear that oxygen-centred free-radical formation associated with human neutrophils is more complex than originally perceived. Understanding the chemistry of these events is critical to our concept of the microbialic process and the development of strategies aimed at preventing damage resulting from neutrophil-derived oxidants.

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REFERENCES

Neutrophil degranulation inhibits potential hydroxyl-radical formation


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