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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

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Hydroxyl radical ('OH) formation by neutrophils *in vitro* requires exogenous iron. Two recent studies [Britigan, Rosen, Thompson, Chai & Cohen (1986) J. Biol. Chem. **261**, 17026–17032; Winterbourn (1987) J. Clin. Invest. **78**, 545–550] both reported that neutrophil degranulation could potentially inhibit the formation of 'OH, but differed in their conclusions as to the responsible factor, myeloperoxidase (MPO) or lactoferrin (LF). By using a previously developed spin-trapping system which allows specific on-line detection of superoxide anion (O_2^-) and 'OH production, the impact of MPO and LF release on neutrophil 'OH production was compared. When iron-diethylenetriaminepenta-acetic acid-supplemented neutrophils were stimulated with phorbol myristate acetate or opsonized zymosan, 'OH formation occurred, but terminated prematurely in spite of continued O_2^- generation. Inhibition of MPO by azide increased the magnitude, but not the duration, of 'OH formation. No azide effect was noted when MPO-deficient neutrophils were used. Anti-LF antibody increased both the magnitude and duration of 'OH generation. Pretreatment of neutrophils with cytochalasin B to prevent phagosome formation did not alter the relative impact of azide or anti-LF on neutrophil 'OH production. An effect of azide or anti-LF on spin-trapped-adduct stability was eliminated as a confounding factor. These data indicate that neutrophils possess two mechanisms for limiting 'OH production. Implications for neutrophil-derived oxidant damage are discussed.

INTRODUCTION

The microbicidal and/or inflammatory activity of human neutrophils is linked to their ability to form superoxide anion (O_2^- [1]) and H_2O_2 [2]. H_2O_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 investigators 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 vivo* 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-leucylphenylalanine; PMA, phorbol myristate acetate; Me_2SO, dimethyl sulphoxide; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; HBSS, Hanks balanced salt solution without Phenol Red; DMPO-OOH, 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl; DMPO-OH, 2,2,-dimethyl-5-hydroxy-1-pyrrolidinyloxyl; 'CH_3, methyl radical; DMPO-CH_3, 2,2,5-trimethyl-1-pyrrolidinyloxyl; N_3^-, azide (NaN_3).

that myeloperoxidase (MPO) released during neutrophil stimulation could inhibit the magnitude of ·OH formed (thiobarbituric acid-reactive 2-deoxyribose oxidation products) by the addition of iron–EDTA to a xanthine/xanthine oxidase O_2^--generating system. This presumably occurred as a consequence of MPO removing H_2O_2 and/or O_2^- from the system [40,41]. Although these data suggested release of MPO would also inhibit formation of ·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 ·OH formation.

This work was presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy held in New York on 6 October 1987 (and published in part in the abstracts) and at a meeting of the Midwest Section of the American Federation for Clinical Research held in Chicago on 11 November 1988.

MATERIALS AND METHODS

Reagents

Diethylenetriaminepenta-acetic acid (DTPA), cytochalasin B, ferricytochrome *c*, hypoxanthine, xanthine oxidase, superoxide dismutase (SOD), catalase, *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 sulphoxide (Me_2SO) 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 (0.9% NaCl) suspended (30 mg/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, Cochransville, PA., U.S.A.

Neutrophil separation

Blood was drawn from healthy volunteers into heparinized syringes. Plasmagel (Roger Bellon, Neuilley, France [43]) or dextran sedimentation [44] was employed to separate leucocytes and erythrocytes. Experimental results were independent of the method of cell separation. The leucocyte-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 μg/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 *o*-dianisidine MPO assay and Western-blot analysis [45].

O_2 consumption

O_2 consumption was measured in a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Neutrophils [$(0.5–1.5) \times 10^7$/ml] were incubated in 1 ml of HBSS at 37 °C. After the addition of the desired stimulus, O_2 saturation was recorded continuously and results were expressed as maximal O_2 consumption rate (nmol/min) observed [43]. Azide (N_3^-; 1–10 mM) increased PMA- and opsonized-zymosan-stimulated 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 [$(1–20) \times 10^6$/ml] were placed in 1 ml of HBSS containing ferricytochrome *c*, (80 μM), with or without cytochalasin B (5 μg/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^{-5} M), absorbance was recorded continuously at 550 nm and 25 °C. DMPO did not affect ferricytochrome *c* reduction by 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 [$(0.5–2.0) \times 10^7$/ml], DMPO (0.1 M), Me_2SO (0.14 M or 0.28 M), DTPA (0.1 mM), with or without iron (0.1 mM) 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 3.2×10^4 with a response time of 1 s. Neutrophil stimuli employed were PMA (100 ng/ml), opsonized zymosan (3 mg/ml) or FMLP (10^{-5} 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 μg of IgG/ml) and cytochalasin B (5 μg/ml) alone or in combination. One unit of catalase is defined as that amount of the enzyme which will decompose 1.0 μmol of H_2O_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_2SO 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 O_2^- and ·OH to yield the spin-trapped adducts 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl (DMPO-OOH) and 2,2-dimethyl-5-hydroxyl-1-pyrrolidinyloxyl (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_2SO, ·OH leads to the formation of methyl radical ($\cdot CH_3$), which can be spin-trapped with DMPO as 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3), [19,25–29]. The reaction kinetics of ·OH with DMPO and Me_2SO are similar. Therefore when experimental conditions are created in which the concentration of Me_2SO 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 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_2SO to yield $\cdot CH_3$ [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.

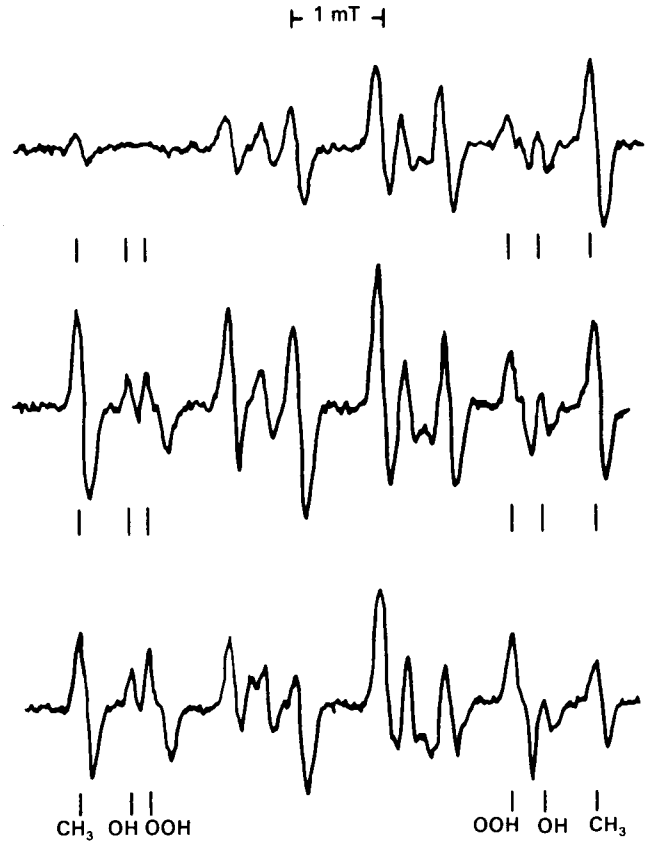

Fig. 1. E.p.r. spectra obtained after PMA stimulation of neutrophils in the presence of exogenous iron

Three sequential e.p.r. spectra (6 min/scan) obtained immediately after PMA stimulation of neutrophils suspended in HBSS containing Me_2SO, DMPO, DTPA and iron (0.1 mM) are shown. The first scan is dominated by DMPO-CH_3, indicating ·OH formation. By the second and third scan, however, DMPO-CH_3 peaks have decreased and DMPO-OOH is the dominant species observed, suggesting cessation of ·OH production in spite of continued O_2^- formation. Spectra are representative of eight separate experiments. High- and low-field peaks corresponding to the spin-trapped adducts DMPO-CH_3, DMPO-OH, and DMPO-OOH are indicated by 'CH_3', 'OH' and 'OOH' respectively.

When neutrophils were stimulated with PMA in the presence of DMPO, Me_2SO, 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 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 O_2^--derived spin-trapped adducts suggested that decreasing 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. N_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 N_3^- and anti-LF yielded scans identical with that obtained with anti-LF alone (results not shown).

N_3^- also inhibits catalase activity [49]. Given the importance of the concentration of H_2O_2 to the Haber–Weiss reaction, experiments were performed to eliminate the possibility that inhibition of neutrophil catalase was responsible for the effect of N_3^- observed. Neutrophils which were totally MPO-deficient were stimulated with PMA in the presence of iron, and the effect of N_3^- was assessed. As shown in Fig. 3, N_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-

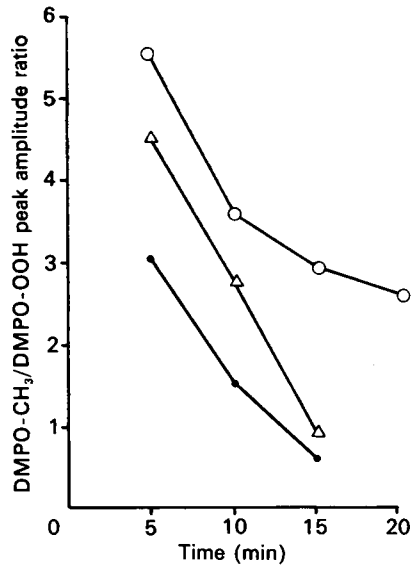

Fig. 2. Effect of N_3^- and anti-LF on the magnitude and duration of 'OH production after PMA stimulation of iron-supplemented neutrophils

The changes in the ratio of high- and low-field DMPO-CH_3 and DMPO-OOH peak amplitudes observed in sequential e.p.r. scans (see Fig. 1) obtained after PMA stimulation of neutrophils in the presence of DMPO, Me_2SO, DTPA and iron are shown (●). Over the 18 min period DMPO-CH_3 production waned in spite of continued DMPO-OOH accumulation. Addition of azide (1 mM) to the neutrophil system (△) increased the initial magnitude (greater DMPO-CH_3/DMPO-OOH peak ratio), but not the duration, of apparent 'OH formation (time required for DMPO-CH_3/DMPO-OOH ratio to become < 1). Addition of anti-LF (10 μg/ml) increased both the magnitude and duration of apparent 'OH production (○). Results are representative of five separate experiments.

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-OH 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-OH by a mechanism that remains a subject of controversy [28,39]. Since it was impossible to determine what proportion of DMPO-OH detected resulted from O_2^- as compared with 'OH production, DMPO-OH peak amplitudes were not considered when calculating ratios of 'OH- to O_2^--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^--derived spin-trapped adducts ('O_2^--dominated').

In the presence of N_3^-, 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^--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 M-DMPO inhibited FMLP-stimulated O_2^- production by > 95%. This appears to result from the capacity 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^- 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

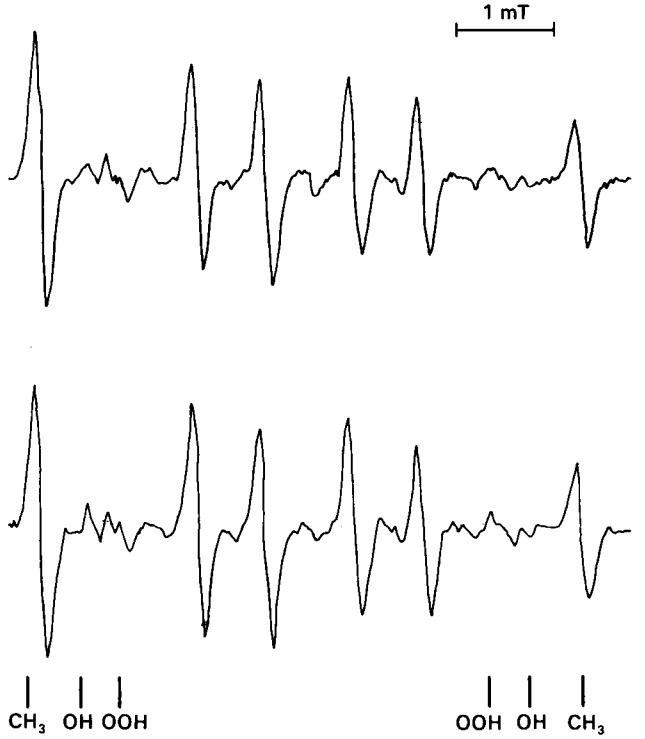

Fig. 3. Effect of N_3^- on 'OH production by iron-supplemented MPO-deficient neutrophils

The top scan was obtained immediately after the addition of PMA to MPO-deficient neutrophils (1.5×10^7/ml) suspended in HBSS containing Me_2SO, DMPO, DTPA and iron. The bottom scan was performed under conditions identical with those used for the top scan, except the reaction mixture also contained 10 mM-N_3^-. N_3^- had no effect on the magnitude or duration (not shown) of DMPO-CH_3 detection. High- and low-field peaks corresponding to DMPO-CH_3, DMPO-OH, and DMPO-OOH are designated as in Fig. 1.

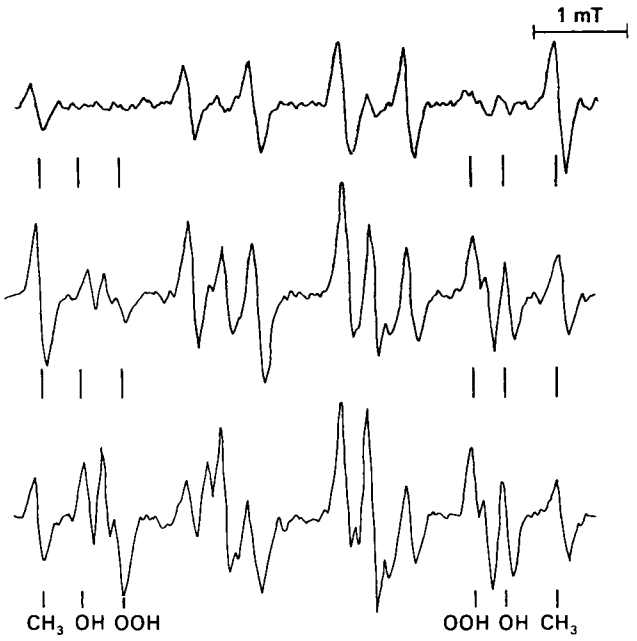

Fig. 4. E.p.r. spectra of opsonized-zymosan stimulation of neutrophils suspended in the presence of exogenous iron

Three sequential (6 min/scan) e.p.r. spectra obtained immediately after the addition of opsonized zymosan (3 mg/ml) to neutrophils (10^7/ml) suspended in HBSS containing Me_2SO, DMPO, DTPA and iron (0.1 mM) are shown. High- and low-field peaks corresponding to DMPO-CH_3, DMPO-OH, and DMPO-OOH are designated as in Fig. 1. As seen with PMA (Figs. 1 and 2), the e.p.r. spectrum initially dominated by DMPO-CH_3 evolved to an O_2^--derived-adduct dominated spectrum (DMPO-OOH/DMPO-OH) over time. Spectra are representative of five 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 N_3^- 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 O_2^- formation [25]. In addition, cytochalasin B decreased the rate of decrease of the DMPO-CH_3/DMPO-OOH peak ratios. This was likely due to the lower rate of O_2^- production, which resulted in a slower accumulation of DMPO-OOH and possibly increased DMPO-CH_3 stability ([39]; see below). As observed above (Fig. 5), N_3^- 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-CH_3, to diamagnetic

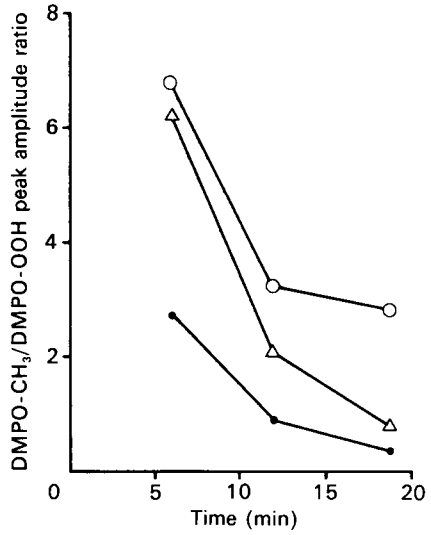

Fig. 5. Effect of N_3^- and anti-LF on the magnitude and duration of ·OH production after opsonized-zymosan stimulation of iron-supplemented neutrophils

Ratios of high- and low-field peaks of DMPO-CH_3 and DMPO-OOH observed over time after opsonzed-zymosan stimulation of neutrophils (10^7/ml) suspended in HBSS containing Me_2SO, DMPO, DTPA and iron (●), as well as 1 mM-N_3^- (△) or anti-LF (10 μg/ml; ○) are shown. N_3^- enhanced the magnitude, but not the duration, of apparent ·OH production, whereas anti-LF increased both. Results are representative of four separate experiments.

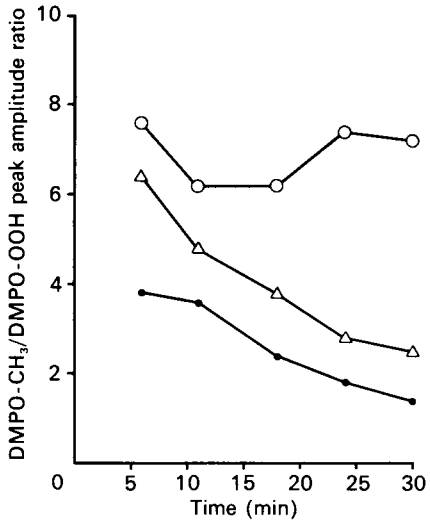

Fig. 6. Effect of N_3^- and anti-LF on ·OH production after opsonized-zymosan stimulation of neutrophils pretreated with cytochalasin B in the presence of exogenous iron

Ratios of high- and low-field DMPO-CH_3 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 Me_2SO, DMPO, DTPA and iron (●) are shown. Addition of 10 mM-N_3^- (△) increased the magnitude of ·OH production (initial increase in DMPO-CH_3/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.

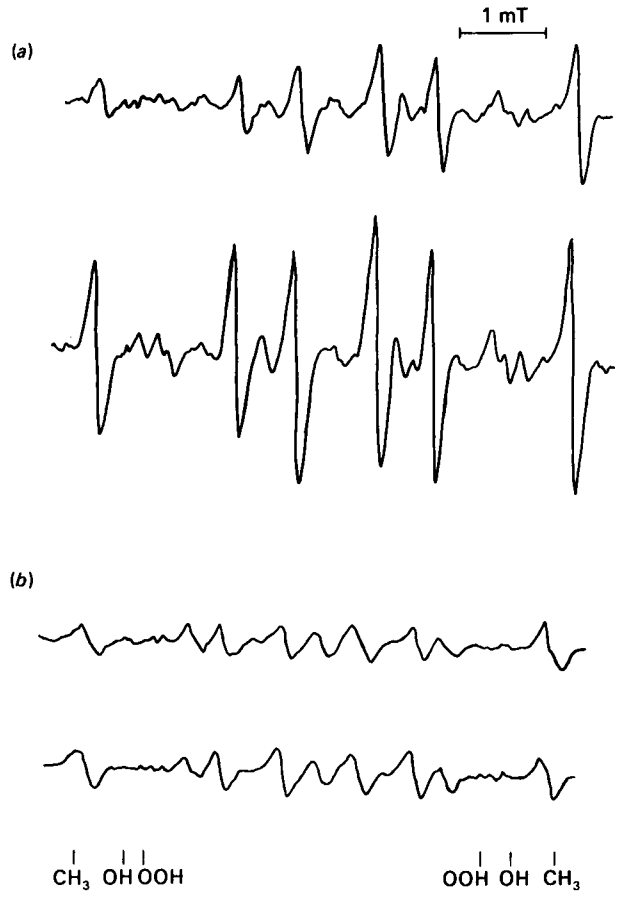

Fig. 7. Effect of SOD on EPR spectra following stimulation of iron-supplemented neutrophils

(a) Two sequential e.p.r. spectra recorded beginning immediately after the addition of SOD (30 units/ml) to neutrophils (5×10^6/ml) suspended in the presence of Me_2SO, 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-CH_3 peaks observed in the presence of SOD. High- and low-field DMPO-CH_3, DMPO-OH and DMPO-OOH peaks designated as in Fig. 1. Results representative of three separate experiments. The receiver gain was 3.2×10^4.

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 N_3^- and/or anti-LF described above could be related to increased DMPO-CH_3 stability rather than increased 'OH production. Several experimental approaches were taken to examine the possibility.

The decrease in DMPO-CH_3 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 O_2^-, 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-CH_3 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-CH_3 peaks (Fig. 7). Addition of anti-LF to stimulated neutrophils had no effect on the rate of neutrophil O_2^- production (O_2 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-CH_3 peaks resulting from stimulation of iron-supplemented neutrophils could not be explained on the basis of increased DMPO-CH_3 stability.

MPO-derived oxidants may convert both DMPO-OH and DMPO-CH_3 into diamagnetic species [53,53a]. However, given the high rate of DMPO-OH and DMPO-CH_3 decomposition occurring as a consequence of neutrophil stimulation [29,39], the relative impact of MPO on spin-trapped-adduct stability was unclear. Small DMPO-CH_3 peaks are observed when neutrophils are stimulated in the absence of iron, owing to DMPO-OOH decomposition to 'OH and its subsequent reaction with Me_2SO to form 'CH_3. Consequently, e.p.r. spectra were obtained after PMA or opsonized-zymosan stimulation of neutrophils suspended in HBSS containing DMPO, Me_2SO and DTPA in the absence or presence of N_3^-. N_3^- had no effect on the peak heights of DMPO-OH or DMPO-CH_3, but increased DMPO-OOH (results not shown), consistent with the known ability of N_3^- to increase neutrophil O_2^- production [54,55]. Also, consistent with a lack of significant effect of MPO on DMPO-CH_3 stability, the rate of DMPO-CH_3 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 O_2^- and H_2O_2, the availability of transition-metal catalysts such as iron, and possibly the ratio of Fe^{3+} to Fe^{2+} [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 O_2^-, 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 O_2 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 e.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 $^{\cdot}OH$ formation. As previously noted [27], the continued accumulation of O_2^--derived spin-trapped adducts eliminated cessation of O_2^-/H_2O_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 $^{\cdot}OH$ scavenger [58] at the concentration employed, it had no effect on the spin trapping of $^{\cdot}OH$ 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 $^{\cdot}OH$ 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 $^{\cdot}OH$ 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-thiomethylbutyric acid [6,7,16], benzoic acid [9–11], Me_2SO [8] and salicylate [12] were offered as evidence for the endogenous capacity of neutrophils to produce $^{\cdot}OH$. 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 $^{\cdot}OH$ 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 $^{\cdot}OH$.

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 $^{\cdot}OH$ formation has previously been shown by demonstrating that cell-free supernatant obtained after neutrophil stimulation inhibited enzymically generated $^{\cdot}OH$ [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 $^{\cdot}OH$ generation by stimulated neutrophils [60,61]. This hypothesis has been questioned extensively [35–38], and it seems unlikely that the conditions required for potential $^{\cdot}OH$ 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 $^{\cdot}OH$ 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 $^{\cdot}OH$ 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 presence 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 e.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 $^{\cdot}OH$ 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 $^{\cdot}OH$ is expected.

Our results therefore suggest that both neutrophil MPO and LF release can inhibit formation of $^{\cdot}OH$

in vitro by iron-supplemented neutrophils. MPO presumably acts by consumption of H_2O_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]. Intraphagosomal MPO may not have any impact on extraphagosmal 'OH production. In fact, if microbial iron is capable of catalysing the Haber–Weiss reaction [65–67], intraphagosomal 'OH production might help to explain the near-normal microbicidal 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 microbicidal process and the development of strategies aimed at preventing damage resulting from neutrophil-derived oxidants.

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REFERENCES

1. Root, R. K. & Cohen, M. S. (1981) Rev. Infect. Dis. **3**, 565–598
2. Root, R. K. & Metcalf, J. A. (1977) J. Clin. Invest. **60**, 1266–1279
3. Haber, F. & Weiss, J. (1934) Proc. R. Soc. London Ser A. **147**, 332–351
4. Beauchamp, C. & Fridovich, I. (1970) J. Biol. Chem. **245**, 4641–4646
5. Weiss, S. J. (1986) Acta Physiol. Scand. Suppl. **548**, 9–37
6. Weiss, S. J., Rustagi, P. K. & LeBuglio, A. F. (1978) J. Exp. Med. **147**, 316–323
7. Tauber, A. I. & Babior, B. M. (1977) J. Clin. Invest. **60**, 374–379
8. Repine, J. E., Eaton, J. W., Anders, M. W., Hoidal, J. R. & Fox, R. B. (1979) J. Clin. Invest. **64**, 1642–1651
9. Sagone, A. L., Jr., Wells, R. M. & DeMocko, C. (1980) Inflammation **4**, 65–71
10. Sagone, A. L., Jr., Decker, M. A., Wells, R. M. & DeMocko, C. (1980) Biochim. Biophys. Acta **628**, 90–97
11. Alexander, M. S., Husney, R. M. & Sagone, A. L., Jr. (1986) Biochem. Pharmacol. **35**, 3649–3651
12. Sagone, A. L. & Husney, R. M. (1987) J. Immunol. **138**, 2177–2183
13. Rosen, H. & Klebanoff, S. J. (1979) J. Clin. Invest. **64**, 1725–1729
14. Green, M. R., Hill, H. A. O., Okolow-Zubkowska, M. J. & Segal, A. W. (1979) FEBS Lett. **100**, 23–26
15. Hawley, D. A., Kleinhans, F. W. & Biesecker, J. L. (1983) J. Clin. Pathol. **79**, 673–677
16. Klebanoff, S. J. & Rosen, H. (1978) J. Exp. Med. **148**, 490–506
17. Tauber, A. I., Gabig, T. G. & Babior, B. M. (1979) Blood **53**, 666–676
18. Pryor, W. A. & Tang, R. H. (1978) Biochem. Biophys. Res. Commun. **81**, 498–503
19. Finkelstein, E., Rosen, G. M. & Rauckman, E. J. (1980) Arch. Biochem. Biophys. **200**, 1–16
20. Winston, G. W., Eibschutz, O. M., Strekas, T. & Cederbaum, A. I. (1986) Biochem. J. **235**, 521–529
21. Green, T. R., Fellman, J. H. & Eicher, A. L. (1985) FEBS Lett. **192**, 33–36
22. Cohen, M. S., Britigan, B. E., Hassett, D. J. & Rosen, G. M. (1988) Free Radical Biol. Med. **5**, 81–88
23. Winterbourn, C. C. (1987) Free Radical Biol. Med. **3**, 33–39
24. Czapski, G. (1984) Isr. J. Chem. **24**, 29–32
25. Britigan, B. E., Rosen, G. M., Chai, Y. & Cohen, M. S. (1986) J. Biol. Chem. **261**, 4426–4431
26. Britigan, B. E., Cohen, M. S. & Rosen, G. M. (1987) J. Leukocyte Biol. **41**, 349–362
27. Britigan, B. E., Rosen, G. M., Thompson, B. Y., Chai, Y. & Cohen, M. S. (1986) J. Biol. Chem. **261**, 17026–17032
28. Rosen, G. M., Britigan, B. E., Cohen, M. S., Ellington, S. P. & Barber, M. J. (1988) Biochim. Biophys. Acta **969**, 236–241
29. Pou, S., Rosen, G. M., Britigan, B. E. & Cohen, M. S. (1989) J. Biol. Chem., **264**, 12299–12302
30. Winterbourn, C. C. (1986) J. Clin. Invest. **78**, 545–550
31. Greenwald, R. A., Rush, S. W., Moak, S. A. & Weitz, Z. (1989) Free Radical Biol. Med. **6**, 385–382
32. Thomas, M. J., Shirley, P. S., Hedrick, C. & DeChatelet, L. R. (1986) Biochemistry **25**, 8042–8048
33. Thomas, M. J., Hedrick, C. & Shirley, P. (1986) In Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine (Rotiolo, G., ed.), pp. 19–21, Elsevier Science Publishers B.V., New York
34. Kaur, H., Fagerheim, I., Grootveld, M., Puppo, A. & Halliwell, B. (1988) Anal. Biochem. **172**, 360–367
35. Baldwin, D. A., Jenny, E. R. & Aisen, P. (1984) J. Biol. Chem. **259**, 13391–13394
36. Winterbourn, C. C. (1983) Biochem. J. **210**, 15–19
37. Aruoma, O. I. & Halliwell, B. (1987) Biochem J. **241**, 273–278
38. Gutteridge, J. M. C., Patterson, S. K., Segal, A. W. & Halliwell, B. (1981) Biochem. J. **199**, 259–261
39. Samuni, A., Black, C. D. V., Krishna, C. M., Malech, H. L., Bernstein, E. F. & Russo, A. (1988) J. Biol. Chem. **263**, 13797–14801
40. Kettle, A. J. & Winterbourn, C. C. (1988) Biochem. J. **252**, 529–536
41. Kettle, A. J., Sangster, D. F., Gebicki, J. M. & Winterbourn, C. C. (1988) Biochim. Biophys. Acta **956**, 58–62

42. Bonnett, R., Brown, R. F. C., Clark, V. M., Sutherland, O. & Todd, A. (1959) J. Chem. Soc. 2094–2101
43. Cohen, M. S. & Cooney, M. H. (1984) J. Infect. Dis. **150**, 49–56
44. Borregaard, N., Heiple, J. M., Simons, E. R. & Clark, R. A. (1983) J. Cell Biol. **97**, 52–61
45. Nauseef, W. M. (1986) Blood **67**, 865–872
46. Cohen, M. S., Metcalf, J. A. & Root, R. K. (1980) Blood **55**, 1003–1010
47. Harris, D. C. & Aisen, P. (1973) Biochim. Biophys. Acta **329**, 156–158
48. McCord, J. M. & Fridovich, I. (1969) J. Biol. Chem. **244**, 6049–6055
49. Keilan, D. & Hartree, E. F. (1934) Nature (London) **134**, 933–934
50. Wang-Iverson, P., Pryzwansky, K. B., Spitznagel, J. K. & Cooney, M. H. (1978) Infect. Immun. **22**, 945–955
51. Pryzwansky, K. B., Martin, L. E. & Spitznagel, J. K. (1978) J. Reticuloendothelial Soc. **24**, 295–310
52. Pou, S., Rosen, G. M., Britigan, B. E. & Cohen, M. S. (1989) Biochim. Biophys. Acta **991**, 459–464
53. Janzen, E. G., Jandrisits, L. T. & Barber, D. L. (1987) Free Radical Res. Commun. **4**, 115–124
53a. Britigan, B. E. & Hamill, D. R. (1989) Arch. Biochem. Biophys., in the press
54. Nauseef, W. M., Metcalf, J. A. & Root, R. K. (1983) Blood **61**, 483–492
55. Klebanoff, S. J. & Hamon, C. B. (1972) J. Reticuloendothelial Soc. **12**, 170–196
56. Minotti, G. & Aust, S. D. (1987) J. Biol. Chem. **262**, 1098–1104
57. Aruoma, O. I., Halliwell, B., Laughton, M. J., Quinlan, G. J. & Gutteridge, J. M. C. (1989) Biochem. J. **258**, 617–620
58. Klebanoff, S. J. (1982) Biochemistry **21**, 4110–4116
59. Ainscough, E. W., Brodie, A. M., Plowan, J. E., Bloor, S. J., Loehr, J. S. & Loehr, J. M. (1980) Biochemistry **19**, 4072–4079
60. Ambruso, D. R. & Johnston, R. B., Jr. (1981) J. Clin. Invest. **67**, 352–360
61. Bannister, J. V., Bannister, W. H., Hill, H. A. O. & Thornalley, P. J. (1982) Biochim. Biophys. Acta **715**, 116–120
62. Babior, B. M., Kipnes, R. S. & Curnutte, J. T. (1973) J. Clin. Invest. **52**, 741–744
63. Britigan, B. E., Coffman, T. J., Adelberg, D. R. & Cohen, M. S. (1988) J. Exp. Med. **168**, 2367–2372
64. Parry, M. F., Root, R. K., Metcalf, J. A., Delaney, K. K., Kaplow, L. S. & Richar, W. J. (1981) Ann. Intern. Med. **95**, 293–301
65. Repine, J. E., Fox, R. B. & Berger, E. M. (1981) J. Biol. Chem. **256**, 7094–7097
66. Repine, J. E., Johansen, K. S. & Berger, E. M. (1984) Infect. Immun. **43**, 435–437
67. Cohen, M. S., Chai, Y., Britigan, B. E., Eagan, T. & Rosen, G. M. (1985) Program and Abstracts of the Twenty-Fifth Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, p. 91, American Society for Microbiology, Washington
68. Gallin, J. I. (1985) Annu. Rev. Med. **36**, 263–274
69. Thomas, C. E., Morehouse, L. A. & Aust, S. D. (1985) J. Biol. Chem. **260**, 3275–3280
70. Biemond, P., van Eijk, H. G., Swaak, A. J. G. & Koster, J. F. (1984) J. Clin. Invest. **73**, 1576–1579
71. Sadrzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E. & Eaton, J. W. (1984) J. Biol. Chem. **259**, 14354–14356
72. Gannon, D. E., Varani, J., Phan, S. H., Ward, J. H., Kaplan, J., Till, G. O., Simon, R. H., Ryan, V. S. & Ward, P. A. (1987) Lab. Invest. **57**, 37–44
73. Coffman, T. J., Cox, C. D. & Britigan, B. E. (1989) Clin. Res. **37**, 426A

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