Intracellular Singlet Oxygen Generation by Phagocytosing Neutrophils in Response to Particles Coated with a Chemical Trap*

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To determine if singlet oxygen ($O_2^\left(\Delta_s\right)$) is produced by neutrophils (PMNs) during the process of phagocytosis, glass beads were coated with a specific chemical trap for $O_2^\left(\Delta_s\right)$, 9,10-diphenylanthracene (DPA). Singlet oxygen, but not other reactive oxygen species, reacted with DPA at a rate of $k = 1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ to form a stable product, DPA-endoperoxide (Corey, E. J., and Taylor, W. C. (1964) J. Am. Chem. Soc. 86, 3881-3882; Wasserman, H. H., Scheffer, J. R., and Cooper, J. L. (1972) J. Am. Chem. Soc. 94, 4991-4996; Turro, N. J. (1972) Am. Chem. Soc. 94, 2671-2677). The production of DPA-endoperoxide was determined by ultraviolet spectroscopy as a decrease in DPA absorbance at 355 nm. The absorbance of DPA was normalized to the absorbance of perylene, which was included in the coating on the beads as a nonreactive, internal standard. In the present study, DPA- and perylene-coated beads were initially allowed to adhere to fibroblast-coated coverslips. PMNs were then added to the bead-coated coverslips and allowed to adhere and phagocytose the beads for 1 h at 37°C. In some experiments, 4B-phorbol-12-myristate-13-acetate (PMA) (1 ng/2.5 x 10^6 cells/ml), a known activator of the PMN oxidase, was added as a co-stimulant. The amount of $O_2^\left(\Delta_s\right)$ produced by phagocytically stimulated PMNs was calculated to be 11.3 ± 1.25 pmol of $O_2^\left(\Delta_s\right)/1.25 \times 10^6$ cells. Low dose PMA co-stimulation increased the production of $O_2^\left(\Delta_s\right)$ to 14.1 ± 4.1 pmol/1.25 x 10^6 cells. Averaged together these amounts represent approximately 19 ± 5.0% of the total oxygen consumed by PMNs in response to DPA- and perylene-coated beads. The specificity of the DPA reaction with $O_2^\left(\Delta_s\right)$ was confirmed by warming to 120°C, which releases $O_2^\left(\Delta_s\right)$ from the DPA-endoperoxide, regenerating the parent DPA compound (Wasserman et al., 1972; Turro et al., 1981) and the absorbance at 355 nm. In addition, δ-carotene, an avid quencher of singlet oxygen, was included in the coating of some bead preparations; assays in which these beads were used showed no change in the absorbance at 355 nm. Singlet oxygen production by myeloperoxidase was also measured using the coated bead assay and the results suggest that this is a major pathway by which singlet oxygen is generated in phagocytically stimulated PMNs. The formation of $O_2^\left(\Delta_s\right)$ by PMNs has been suggested by others; however, previous methods for $O_2^\left(\Delta_s\right)$ detection have either been nonspecific, insensitive, or incapable of measuring the intracellular production of $O_2^\left(\Delta_s\right)$. With the use of DPA- and perylene-coated beads, we have developed a specific and sensitive method for the intracellular detection of $O_2^\left(\Delta_s\right)$ and have demonstrated that $O_2^\left(\Delta_s\right)$ is produced during the process of phagocytosis.

The respiratory burst is a characteristic feature of neutrophils and other phagocytes and is essential for the defense of an organism against invading microorganisms (for review see Babior (1978) and Badawy and Karnovsky (1980)). The respiratory burst reflects the activation of a transmembrane enzyme system, NADPH:O2-oxidoreductase (commonly called NADPH-oxidase), that transfers one electron from the cytosolic donor NADPH to a molecule of extracellular oxygen, producing superoxide anion ($O_2^-$) (Rossi and Zatti, 1964; Babior et al., 1973; Goldstein et al., 1977; Green et al., 1980; Babior et al., 1981; Clark et al., 1987). NADPH-oxidase derived $O_2^-$ is further transformed into reactive oxygen species which include hydrogen peroxide (H2O2) (Iyer et al., 1961; Paul and Sbarra, 1968), singlet oxygen ($O_2^\left(\Delta_s\right)$) (Allen et al., 1972; Krinsky, 1974; Rosen and Klebanoff, 1979), hydroxyl radicals (·OH) (Tauber and Babior, 1977), and hypochlorous acid (HOCl) (Harrison and Shultz, 1976; Weis et al., 1982). HOCl is produced in the presence of Cl- and H2O2 by myeloperoxidase (MPO), an enzyme active in the phagolysosomes of PMNs (Agner, 1941; Bainton and Farquhar, 1968). The importance of $O_2^-$ and H2O2 production for PMN bactericidal activity is well known since a lack of production of $O_2^-$ and H2O2 by PMNs from patients with chronic granulomatous disease (Holmes et al., 1967; Quie et al., 1967; Babior et al., 1975; Curnutte and Babior, 1974) results in an increased susceptibility to life-threatening bacterial infections. These and many other studies have suggested that the actual bactericidal agents are products formed by the dismutation of $O_2^-$ or the interaction of $O_2^-$ and H2O2 (Haber-Weiss reaction), as well as products generated by the MPO/H2O2/C1- system; less frequently bactericidal activity has been attributed to $O_2^-$ or H2O2 directly (for review see Klebanoff, 1988). Most of these studies, however, did not involve the direct measurement of $O_2^\left(\Delta_s\right)$ or ·OH production by stimulated PMNs.

To determine what toxic oxygen products are generated by

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The abbreviations used are: $O_2^-$, superoxide anion; $O_2^\left(\Delta_s\right)$, singlet oxygen; DPA, 9,10-diphenylanthracene; MPO, myeloperoxidase; H2O2, hydrogen peroxide; ·OH, hydroxyl radical; HOCl, hypochlorous acid; PBS, phosphate-buffered saline; PMA, 4B-phorbol-12-myristate-13-acetate; PMNs, polymorphonuclear leukocytes; FPLC, fast programmed liquid chromatography; HPLC, high performance liquid chromatography.

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Singlet Oxygen Production by PMNs

phagocytically stimulated PMNs, we have focused in the present study on the production of \(O_2(1\Delta_g)\). Singlet oxygen \((1\Delta_g)\) is a highly reactive, diffusing, and long-lived electronically excited state of molecular oxygen (Wilson and Hastings, 1970; Khan and Kashia, 1970; Browne and Ogryzlo, 1965; Corey and Taylor, 1964; Foote and Wexler, 1964). Highly efficient mechanisms for its generation exist that involve photosensitization of the ground triplet state of oxygen \((\Sigma_g^+)\), electron transfer from \(O_2\), thermal dissociation of endoperoxides, and chemical reactions involving \(H_2O_2\) and hypohalites. Despite known mechanisms for its generation, controversies still exist as to the extent of \(O_2(1\Delta_g)\) production by intact cells. The controversies in part arise from the use of both nonspecific and insensitive methods for the measurement of \(O_2(1\Delta_g)\). Previous methods using visible spectrum chemiluminescence (Allen et al., 1972; Allen, 1975) or furan (Rosen and Klebanoff, 1977) as a chemical trap for \(O_2(1\Delta_g)\) have provided information consistent with the generation of \(O_2(1\Delta_g)\) by PMNs, but both methods relied on the use of nonspecific techniques to determine the products formed from the reduction or excitation of molecular oxygen (Takayama et al., 1977; Held et al., 1978; Harrison et al., 1978). A specific chemical method for the detection of \(O_2(1\Delta_g)\) was developed by Kulig and Smith (1973) which established the reactivity of cholesterol with \(O_2(1\Delta_g)\) and identified a specific product of \(O_2(1\Delta_g)\) as a \(O_2(1\Delta_g)\) trap estimated that \(\pm 0.2\%\) of the oxygen taken up by PMNs during phagocytosis was excited to \(O_2(1\Delta_g)\). Although specific, this method is very insensitive since the reaction between \(O_2(1\Delta_g)\) and cholesterol is very slow, \(k = 8.44 \times 10^9\) M\(^{-1}\) s\(^{-1}\) (Schneck et al., 1957). Even more recently, an ultrasensitive near-IR emission spectrophotometer was developed by Khan and Kashia (1979) that specifically detects the relaxation emission of \(O_2(1\Delta_g)\) at 1286 nm (Browne and Ogryzlo, 1965) as it returns to ground state molecular oxygen. This spectrophotometer has been used to establish the chemical production of \(O_2(1\Delta_g)\), as well as its production in purified enzyme systems (for review see Kanofsky (1989) and Khan (1991)). Kanofsky et al. (1988), using a modification of this instrument to kinetically measure the production of \(O_2(1\Delta_g)\), have recently published that isolated eosinophil peroxidase in the presence of \(Br^-\) produces \(O_2(1\Delta_g)\), although PMA-stimulated eosinophile generated very little \(O_2(1\Delta_g)\) in comparison. The apparent discrepancy in the ability of the eosinophil peroxidase to generate \(O_2(1\Delta_g)\) and the attempts to measure its production in intact eosinophils is a problem inherent in systems that are capable of generating large amounts of \(O_2\); Superoxide anion is both a source and an efficient quencher of \(O_2(1\Delta_g)\) when present at high concentrations (Khan, 1970, 1977, 1978, 1981; Nilsson and Kears, 1974; Rosenthal, 1975; Guiraud and Foote, 1976; Corey et al., 1987). The use of PMA (10 \(\mu\)g/10\(^6\) cells) as a stimulus leads to an enormous generation of \(O_2\) compared with the amounts of \(O_2\) generated in response to more physiologically relevant stimuli such as opsonized bacteria or soluble stimuli such as formyl-methionyl-leucyl-phenylalanine. Kanofsky et al. (1988) also point out several inherent problems in using direct emission spectroscopy to measure \(O_2(1\Delta_g)\) production by intact cells. 1) To measure \(O_2(1\Delta_g)\) it must be released from the cells, since \(O_2(1\Delta_g)\) emission inside the phagolysosome will be optically blocked by the high optical density; 2) to stimulate the release of the peroxidase from cells, high concentrations of PMA were required (which also stimulates the release of large amounts of \(O_2\)); 3) the released \(O_2(1\Delta_g)\) encounters an aqueous environment rich in cellular targets which would result in the immediate quenching of a large fraction of the \(O_2(1\Delta_g)\) produced and reduce the emission signal below the sensitivity of the spectrophotometer. Kanofsky et al. (1988) have also reported that purified MPO from PMNs did not produce significant amounts of \(O_2(1\Delta_g)\) under assay conditions that were required for detection by emission spectroscopy, and that PMA-stimulated PMNs made even less \(O_2(1\Delta_g)\) than eosinophils. Unfortunately, emission spectroscopy is not yet sensitive enough to measure \(O_2(1\Delta_g)\) production by intact cell systems or under conditions that are optimal for MPO activity (Kettle and Winterbourn, 1989; Zuurbier et al., 1990). Therefore, the question still remained unanswered as to whether or not intact PMNs produce \(O_2(1\Delta_g)\).

The purpose of the present study was to develop a specific and sensitive assay that could be used to measure the amount of intracellular \(O_2(1\Delta_g)\) produced in phagocytically stimulated PMNs. The development of the present method was based on earlier findings that established the reactivity rates of \(O_2(1\Delta_g)\) with perylene and DPA and identified the product formed in the reaction of \(O_2(1\Delta_g)\) with DPA (Wasserman et al., 1972; Koo and Schuster, 1978, Turro et al., 1981). Two approaches were taken in the present study using DPA- and perylene-coated glass beads: 1) PMNs were stimulated with the coated beads in the presence and absence of low dose PMA, a known activator of the NADPH-oxidase, and 2) the MPO/H\(_2\)O\(_2)/Cl\(^-\) system was evaluated at pH 7.4 using 30–90 \(\mu\)M H\(_2\)O\(_2\). This is the first reported evidence that PMNs produce a measurable amount of \(O_2(1\Delta_g)\) and that the MPO/H\(_2\)O\(_2)/Cl\(^-\) system in the presence of phagolysosomal concentrations of H\(_2\)O\(_2\) generates \(O_2(1\Delta_g)\).

MATERIALS AND METHODS

Elicited neutrophils from female Hartley guinea pigs \(^2\) (400–500 g) (Elm Hill Breeding Laboratories, Chelmsford, MA) were prepared by the method of DePierre and Karnovsky (1974), using 9% sterile sodium caseinate (Fisher Scientific, Fairlawn, NJ). The cells were further purified over Ficoll-Paque (Pharmacia LKB Biotechnology Inc.) to \(\geq 85\%\) neutrophils, washed twice, and resuspended in phosphate-buffered saline (PBS) (158 mM NaCl, 2.7 mM KCl, 16.2 mM Na\(_2\)HPO\(_4\), 1.47 mM KH\(_2\)PO\(_4\), 0.9 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), and 7.5 mM D-glucose, pH 7.35). Viability of these cells was always \(\geq 90\%\).

Oxygen Consumption by PMNs

Oxygen consumption was measured using a biological oxygen monitor (YSI Inc., model 5300) fitted with a Clark-type oxygen electrode. The measurements were made at 37°C in an airtight bath assembly (YSI Inc., model 5300) under constant stirring with a magnetic stirrer. Oxygen consumption by a suspension of 1.25 \(\times\) 10\(^6\) PMNs incubated in the presence and absence of approximately 20–30 DPA- and perylene-coated beads per PMN was measured for 1 h. The same measurements were made for PMNs co-stimulated with PMA. PMA (1 ng) was added to 2.5 \(\times\) 10\(^6\) cells on ice, and the cells were diluted, then added to the appropriate chamber of the bath assembly containing the DPA- and perylene-coated beads. Calibration of the oxygen electrode was done using the phenylhydrazine oxidation method (Miura and Fridovich, 1976). PMN viability was 89 ± 2.0% after a 60-min incubation with or without DPA/perylene-coated beads.

\(^2\) Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the committee on Care and Use of Laboratory Animal Resources, National Research Council.
Myeloperoxidase Activity Assay

Purified myeloperoxidase (MPO) activity and the MPO activity of 1.25 X 10^6 PMNs were determined using the previously published o-dianisidine method (Dewald and Baggioni, 1986) with the following modifications: 1) the assay was performed at both pH 5.5 and pH 7.4, 2) the o-dianisidine dihydrochloride was used at a final concentration of 52 mM, 3) the reaction was carried out for 5 min at 37 °C in a shaking waterbath and 3) assays were performed in PBS containing D2O and/or H2O. The PMNs were disrupted in PBS containing 0.5% Triton X-100 at 4 °C with a magnetic stirrer and the nuclei and unbroken cells were removed as a pellet by centrifugation. The MPO activity of the postnuclear supernatant was then determined. The range of H2O concentration was 0-120 μM.

Flow Programmed Liquid Chromatography (FPLC) Reverse Phase Column (C18/C8)

DPA and perylene in a 10:1 ratio were coated by evaporation onto the surface of glass tubes and were photosensitized to generate O2(1A2). The coated tubes containing 0.5 ml of PBS-D2O were irradiated with a 100-watt G.E. tungsten lamp at a distance of 6 inches for 5 h at 4 °C with constant slow bubbling of O2. After 5 h of light exposure, the compounds were extracted from the glass using 4.5 ml of redistilled chloroform/methanol (2:1). The chloroform layer containing the DPA, perylene, and any reaction products was removed, evaporated, and redissolved in acetone. This mixture in a volume of 50 μl was applied to a FPLC reverse phase column (C18/C8) and eluted using an acetone/H2O gradient of 66.7-98% (flow rate 1.0 ml/min for DPA, and 1.5 ml/min for perylene, and the reaction product was removed. Elution was monitored at 210 nm, since structurally DPA-endoperoxide could be expected to have a wavelength ultraviolet absorbance shorter than that of DPA, and a significant increase in sample absorbance at 210 nm was observed after light exposure. The retention time for the individual compounds were used to identify the DPA and perylene peaks in the nonreacted and photosensitized samples.

Additional Procedures

The following procedures were carried out in a darkened room. DPA- and Perylene-coated Glass Bead Preparation—Glass beads (1.6 ± 0.3 μm) (Duke Supply, Palo Alto, CA) were coated with perylene and DPA. Perylene (0.75 mg) and 7.5 mg of DPA were dissolved in 1.0 ml of dichloromethane. Beads (3 X 10^16 mg) were prewetted in dichloromethane and then coated with 1 ml of the perylene/DPA mixture by evaporating off the dichloromethane in a chemical hood at room temperature in a sonication waterbath. The coated beads were resuspended in PBS, resulting in an approximate 333 X 10^6 beads determined by weight, and the bead number was estimated to be 1.5 X 10^10/ml PBS. The final amounts of DPA and perylene on 1.5 X 10^10/ml beads was calculated to be 0.014 mg of DPA and 0.0173 mg of perylene as determined by absorbance spectroscopy using their respective extinction coefficients at 355 and 436 nm. β-carotene was added to each bead preparation at an amount equivalent to perylene.

Fibronectin-coated Glass Coverslips (22 mm) —Streptavidin coverslips were placed in six-well tissue culture plates. In a sterile hood, 250 μl of sterile 25 °C PBS containing 2.5 μg of fibronectin was added to each coverslip (Vaudaux et al., 1985). The coverslips were then incubated at 37 °C in a humidified incubator with 5% CO2 for 1 h. The non-adhered fibronectin and liquid were removed and the coverslips were washed 2 times with 25 °C PBS; the coverslips were kept moist and at 4 °C until use.

Phagocytosis by Neutrophils Adhered to Fibronectin-coated Surfaces—In the dark, beads (4-6 X 10^10/ml) coated with perylene and DPA in 250 μl of 25 °C PBS, pH 7.35, were added to each coverslip in six-well tissue culture plates covered with aluminum foil. The coverslips were incubated in the dark for 15 min at 37 °C in a humidified incubator, 5% CO2 atmosphere. The excess liquid, beads, and fluid were removed by gentle aspiration after incubation. PMNs were resuspended in ice-cold PBS in propylene tubes to 5 X 10^6 cells/ml just before use. When present, PMA was added in a 2.5-μl volume to 1 ml of 2.5 X 10^6 PMNs; the cells were gently mixed for 30 s and then diluted to 5 X 10^6/ml in ice-cold PBS. Unstimulated or PMA-stimulated PMNs (250 μl at 5 X 10^6 cells/ml were added to each coverslip and incubated for 1 h at 37 °C (e.g., 1.25 X 10^6 PMNs/coverslip or approximately 20-30 beads/PMN). PMN viability was determined under identical assay conditions using trypan blue before and after the cells were incubated with the coated beads. The nonadherent cells and excess liquid were removed by gentle aspiration and the coverslips were washed 3 X with 250 μl of 25 °C PBS. To extract the perylene and DPA from beads alone or beads ingested by PMNs, three identically treated coverslips were added to 1 ml of conical glass tubes containing 0.08 ml of PBS and 4.5 ml of ice-cold chloroform/methanol (2:1). The tubes were sealed with Teflon-lined caps, vortexed for 1 min, and centrifuged at 4 °C, 400 X g, for 5 min. Perylene and DPA were recovered in the lower chloroform layer, and 3 ml were placed in a quartz cuvette and an absorbance spectrum from 500 to 300 nm was recorded using a scanning spectrophotometer with chart recorder (Beckman Instruments). Five major peak absorbances were observed and monitored. 1) The first peak was a specific perylene absorbance peak at 438 nm, 2) the next two major peaks were at 395 and 376 nm which represent the combined absorbances of perylene at 412 and 390 nm and DPA at 392 and 372 nm, and 3) two independent DPA absorbance peaks at 335 and 340 nm.

To obtain a change in absorbance (ΔO.D.) for 1.25 X 10^6 PMNs, three coverslips were combined since the average number of cells per coverslip was 4 ± 0.2 X 10^6 determined by light microscopy. A decrease in the absorbance at 355 nm was used to calculate the amount of DPA-endoperoxide formed, which is proportional to the amount of O2(1A2) produced. The ΔO.D. at 355 nm or the amount of DPA-endoperoxide generated can be determined by multiplying the initial absorbance of DPA at 355 nm by the ratio of the initial perylene absorbance at 438 nm divided by the final absorbance of perylene and subtracting the final absorbance of DPA [DPAfinal = (perylene(0.004) - DPA-endoperoxide generated can be determined by multiplying the parent DPA compound would be regenerated). The samples were then warmed in tightly capped (Teflon-lined) 5 ml glass vials under nitrogen to 120 °C for 1.5 h (conditions under which a majority of the parent compound would be regenerated). The samples were cooled and a second absorbance spectrum from 500 to 300 nm was recorded for each sample using a tetralin reference and a scanning spectrophotometer with chart recorder. The samples were then warmed in tightly capped (Teflon-lined) 5 ml glass vials under nitrogen to 120 °C for 1.5 h (conditions under which a majority of the parent compound would be regenerated). The samples were cooled and a second absorbance spectrum from 500 to 300 nm was recorded against a warmed and cooled tetralin reference.

Scanning Electron Microscopy—PMNs were added to fibronectin-coated coverslips containing DPA- and Perylene-coated beads and were allowed to phagocytose for 60 min at 37 °C in 5% CO2 humidified atmosphere. The coverslips were washed 3 times with room temperature PBS and then fixed for 30 min in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After fixation, the cells were washed 3 times in 0.1 M sodium cacodylate buffer (pH 7.4), and then washed with an overnight incubation at 4 °C. The cells were then postfixed in osmium (1% OsO4) for 30 min at 4 °C, washed three times in 0.1 M sodium cacodylate buffer, pH 7.2, then dehydrated in a graded ethanol series. Cells were critical point dried (Balzers Union Limited, Flurstenfurt, Liechtenstein) out of ethanol and coated with gold-palladium (Sputter Coating Sputter Coating, Technics, Inc., Alexandria, VA). Samples were examined in an etec autoscan operated at 20 kV.

Fluorescent Microscopy—Visualization of the DPA/perylene-coated beads and PMNs co-inkubated with beads was achieved by epifluorescence with a Zeiss Axioskop (Oberkochen, Germany). These compounds were observed to have a yellow-green fluorescence (Ex365 excitation filter, LP420 barrier filter). A 100-μl aliquot of coated beads in PBS was examined under a glass coverslip. Approximately 90% of the beads in each preparation were fluorescent. PMNs were not allowed to phagocytose 100 X 10^6 and perylene-coated beads on fibronectin-coated coverslips at 60 min at 37 °C. Cells were washed 3 times with room temperature PBS following the incubation and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at 25 °C. Coverslips with fixed cells were inverted onto a drop of PBS which was ringed by a thin circle of petroleum jelly to prevent drying. Coverslips were examined with a simultaneous epifluorescence filter, LP420 barrier filter. A 100-μl aliquot of coated beads in PBS was examined under a glass coverslip. Approximately 90% of the beads in each preparation were fluorescent. PMNs were not allowed to phagocytose 100 X 10^6 and perylene-coated beads on fibronectin-coated coverslips at 60 min at 37 °C. Cells were washed 3 times with room temperature PBS following the incubation and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at 25 °C. Coverslips with fixed cells were inverted onto a drop of PBS which was ringed by a thin circle of petroleum jelly to prevent drying. Coverslips were examined with a simultaneous epifluorescence filter, LP420 barrier filter.
RESULTS

Reactivity of DPA and Perylene with O₂(1Δg) — The reaction rates of photochemically generated O₂(1Δg) with aromatic hydrocarbons and the reversibility of some of the photosensitized oxidations by warming are well established (e.g. DPA) (Wasserman et al., 1972; Turro et al., 1981). Development of the present method to measure O₂(1Δg) production using DPA and the use of perylene as an internal standard was based on these earlier findings. In brief, it is known that during a photosensitized oxidation, DPA absorbs light energy, is converted to an excited triplet state, and through energy transfer of dried DPA and perylene with O₂(1Δg), since these compounds absorb at 210 and 438 nm, and neither absorbance at 210 nm, decreased when DPA was stored under nitrogen resulted in absorbance of 355 nm and this absorbance, as well as the absorbance at 210 nm, decreased when DPA was reacted with photochemically generated O₂(1Δg) and DPA-endoperoxide was formed. DPA-endoperoxide, due to a loss of ring π-conjugation, absorbs light at shorter wavelengths compared with the parent DPA compound. The present findings indicate that the changes in absorbance at 355 nm can be used to determine the production of O₂(1Δg). These results confirm earlier reports that perylene and DPA have different reactivity rates with O₂(1Δg) and that the reaction of DPA with O₂(1Δg) forms the previously characterized DPA-endoperoxide that can be converted back to the parent DPA compound upon warming.

![Scheme I](image)

**Scheme I**

Singlet  Oxygen  Production  by  PMNs

![Figure 1](image)

**Fig. 1.** FPLC elution profiles of the DPA and perylene before and after photosensitized oxidation. DPA and perylene in a ratio of 10:1 were coated by evaporation onto the interior surface of glass tubes; 0.5 ml of PBS-D₂O was added to the coated tubes and the tubes were irradiated with a 100-watt tungsten lamp for 5 h at 4 °C with constant slow bubbling of O₂. After 5 h of light exposure, the compounds were extracted from the glass using 4.5 ml of ice-cold chloroform/methanol (2:1), the chloroform layer containing the compounds was evaporated under nitrogen, and the compounds were redissolved in acetone. A 50-μl volume of the acetone extraction was applied to an FPLC reverse phase column (C_{18}/C_{8}) and eluted using an acetonitrile/H₂O gradient of 66.7 to 86% (flow rate 1.0 ml/min). The elution was monitored at 210 nm. A, unreacted samples containing DPA and perylene; B, photosensitized sample. Peak 1 of FPLC profile B was further evaluated. C, initial absorbance spectrum in tetralin from 300–500 nm; D, absorbance spectrum after warming to 120 °C for 1.5 h under nitrogen.
Singlet Oxygen Production by PMNs

Efficiency of the Bead Coating Process and Verification of Ingestion of the Coated Beads by PMNs—Glass beads were coated with perylene, DPA, and in some preparations β-carotene, as described under “Materials and Methods.” The coated beads were then resuspended in PBS by weight to 1.6-2.4 × 10⁶/ml. To demonstrate that the glass beads were coated with perylene, DPA, or β-carotene, the beads were examined using fluorescent microscopy. The yellow-green fluorescence of these compounds can be observed at an excitation wavelength of 365 nm using a Hirst filter. Approximately 90% of the beads in each preparation were coated with the compounds as indicated by fluorescence.

To determine whether the DPA- and perylene-coated beads would be ingested by PMNs, 4-6 × 10⁵ beads were added to fibronectin-coated coverslips and allowed to settle and attach for 15 min at 37°C. The nonattached beads were removed, and PMNs (1.25 × 10⁶) were added and incubated on the bead-coated coverslips for 1 h at 37°C. The coverslips were then washed 3× to remove non-cell associated beads and examined using fluorescent microscopy to determine whether the beads were associated with the PMNs and that the non-cell associated beads were removed by washing. A few (<10%) of the PMNs had partially ingested beads on their surfaces that could be easily counted using light microscopy (Nomarski); these beads were highly fluorescent with yellow-green coloration. In addition, beads in lower planes of focus were observed within the PMNs by fluorescent microscopy which were a dimmer blue fluorescence. The plane of focus and the dim blue fluorescence suggests that the beads had been ingested and were not just cell-associated. However, to confirm ingestion of the beads by the PMNs, samples were examined using scanning electron microscopy and ingestion was determined over time from 2.5 to 60 min. At 2.5-5 min the PMNs were actively phagocytosing the beads, by 15 min a large percentage of the beads were ingested, and at 60 min the majority of the beads had been completely ingested and the PMN surface morphology had changed dramatically (Fig. 2). Surface projections normally observed on nonphagocytosing PMNs or at early time points were lost, and the cells took on a more rounded, lumpy appearance. These findings indicate that most of the coated beads were completely ingested by the PMNs and that most of the non-cell-associated beads were removed by washing.

Amount of \( O_2 (\Delta_g) \) Generated by PMNs during Phagocytosis—Having established that the beads were coated with DPA and perylene and that the majority were completely ingested by the PMNs, the coated beads were used in a 1-h phagocytosis assay to determine the amount of \( O_2 (\Delta_g) \) generated by phagocytically stimulated or PMA co-stimulated PMNs. As mentioned above, DPA- and perylene-coated beads were allowed to settle and attach to fibronectin-coated coverslips, and unattached beads were removed. Fibronectin-coated surfaces were used to provide a surface to which the beads and PMNs would attach, and more realistically represent the in vivo conditions under which phagocytosis takes place (Herrmann et al., 1990). PMNs (1.25 × 10⁶) or PMNs co-stimulated in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and prepared for scanning electron microscopy as described under “Materials and Methods” before being examined. A scanning electron micrograph (final magnification ×3500) of PMNs incubated with DPA- and perylene-coated beads for 2.5 min and fixed without washing (A); inset shows an enlargement (∼7000) of a PMN fixed while ingesting a bead (large arrowhead) and containing several already ingested beads (small arrowhead pointing to tear in cell); PMNs incubated with DPA- and perylene-coated beads for 15 min and fixed without washing (∼2000) (B); PMNs added to bead-coated coverslips for 60 min and washed before fixation (∼2000) (C).
with low dose PMA (1.0 ng/2.5 × 10⁶ cells/ml), a known activator of the NADPH-oxidase, were added to the bead-coated coverslips and incubated at 37 °C for 1 h. After incubation, the cells and coated beads were extracted in ice-cold chloroform/methanol and the chloroform layer was removed and scanned using absorbance spectroscopy from 500 to 300 nm. Absorbance spectra of samples containing PMNs plus beads were compared with spectra of similarly incubated beads adhered to fibronectin-coated coverslips. Five distinct absorbance peaks were observed for each sample and all peaks were compared (see Figs. 3 and 4). A decrease or change in the peak absorbance at 355 nm was used to calculate the amount of DPA-endoperoxide formed, which is proportional to the amount of O₂(ΔΔ) produced (Table I). The details and calculations are given under “Materials and Methods.” No change in absorbance at 355 nm was observed when uncoated beads or perylene-coated beads were used in place of beads coated with both DPA and perylene.

To determine if the change in absorbance at 355 nm could be blocked by an avid quencher of O₂(ΔΔ), β-carotene was coated onto some bead preparations (k = 3 × 10⁷ M⁻¹ s⁻¹; 497 nm, 466 absorbance maxima; zero absorbance at 355 nm) (Foote and Denney, 1968; Foote et al., 1970; Mathews-Roth and Krinsky, 1970). In parallel experiments, beads coated with DPA and perylene and beads coated with DPA, perylene, and β-carotene were incubated with PMNs for 1 h on fibronectin-coated surfaces. Samples from PMNs incubated with DPA- and perylene-coated beads showed the typical decrease in absorbance at 355 nm, whereas samples from PMNs incubated with beads also coated with β-carotene showed no change in absorbance at 355 nm (Table I). Taken together, these findings form the basis for using the decrease in absorbance at 355 nm to calculate the amount of O₂(ΔΔ) produced.

**Amount of Oxygen Consumed by PMNs during Phagocytosis—** Oxygen consumption by PMNs in response to coated beads was determined using a Clark oxygen electrode in an air-tight bath assembly at 37 °C under constant stirring with a magnetic stirrer. Oxygen consumption is stoichiometrically related to the amount of O₂ produced by stimulated PMNs, including production within the phagolysosome (Makino et al., 1986). The amount of oxygen consumed by PMNs was calculated using a Clark oxygen electrode in an air-tight bath assembly at 37 °C under constant stirring with a magnetic stirrer. Oxygen consumption is stoichiometrically related to the amount of O₂ produced by stimulated PMNs, including production within the phagolysosome (Makino et al., 1986). The amount of oxygen consumed by PMNs in suspension in response to coated beads or in response to PMA and coated beads is presented in Table I. Averaged together the amount of O₂(ΔΔ) produced by PMNs or PMA co-stimulated PMNs in response to DPA- and perylene-coated beads adhered to fibronectin and represented an estimated 19 ± 5.0% of the total oxygen consumed by PMNs in suspension in response to DPA- and perylene-coated beads or PMA co-stimulation. Based on the previous work of Nathan et al. (1989),
Singlet Oxygen Production by PMNs

it should be recognized that oxygen consumption by PMNs stimulated in suspension may be an underestimation of the amount of oxygen consumed by PMNs adhered to fibronectin.

Verification of the Coated Bead Method to Detect $O_2(\Delta \lambda)$

Production in Stimulated PMNs—The specificity of decreased absorbance at 355 nm representing the reaction of DPA with $O_2(\Delta \lambda)$ can be determined by monitoring for changes in the perylene absorbance peak which would reflect nonspecific reactions and determining the reversibility of the DPA-endoperoxide formation by warming which releases $O_2(\Delta \lambda)$ and regenerates the parent DPA compound (Wasserman et al., 1972; Turro et al., 1981). To reverse the reaction, the chloroform samples were evaporated under nitrogen, redissolved in tetraclin, and warmed to 120°C for 1.5 h. Fig. 4 is a typical absorbance spectrum for a PMN sample before and after warming to 120°C. The absorbance at 355 nm was decreased after the coated beads were reacted with the PMNs for 1 h and is presented in Fig. 4 as the lower absorbance spectrum (lower DPA peaks). When the reacted sample was warmed to 120°C for 1.5 h, the upper absorbance spectrum (higher DPA peaks) was obtained. A similar spectrum was also obtained for unreacted bead samples as the upper absorbance spectrum. No change in the perylene absorbance at 438 nm was observed upon warming; however, the reversal of the decreases in DPA absorbance at 355 nm ranged from 57 to 79%. The reversibility by warming confirms the specificity of the DPA- and perylene-coated beads to detect $O_2(\Delta \lambda)$ production by PMNs. A 100% reversal would not be expected under the present experimental conditions since: 1) warming to 120°C for only 1.5 h will not accomplish the complete conversion of DPA-endoperoxide to DPA (Wasserman et al., 1972), although warming the tetraclin for more than 2 h altered the absorbance spectrum, and 2) some side reactions would be expected in the extracted lipid containing fraction of the PMNs.

Production of $O_2(\Delta \lambda)$ by the Myeloperoxidase/H$_2$O$_2$/Cl$^-$ System—It is well established that myeloperoxidase (MPO) produces HOCl and $O_2^-$ by catalyzing the oxidation of Cl$^-$ by H$_2$O$_2$, but it has only recently been demonstrated that concentrations of H$_2$O$_2$ > 100 μM will result in the inhibition of the MPO/H$_2$O$_2$/Cl$^-$ activity under neutral pH conditions (Kettle and Winterbourn, 1985; Zuurbier et al., 1990). Allen (1972) proposed that the O$_2$ generated in this reaction was $O_2(\Delta \lambda)$, based on the initial discovery by Khan and Kash that a reaction between H$_2$O$_2$ and OCI$^-$ in an aqueous solution leads to the production of $O_2(\Delta \lambda)$ (Khan and Kash, 1963, 1970). The MPO-catalyzed reaction and the reaction of H$_2$O$_2$ and OCI$^-$ are summarized below.

$$H_2O_2 + Cl^- \xrightarrow{MPO} H_2O + HOCl \leftrightarrow OCI^- + H^+ \quad (1)$$

$$H_2O_2 + OCI^- \rightarrow O_2(\Delta \lambda) + H_2O + Cl^- \quad (2)$$

In the present study, the $o$-dianisidine method was used to determine the activity of the MPO/H$_2$O$_2$/Cl$^-$ system of detergent disrupted PMNs (1.25 × 10$^6$) at 37°C using concentrations of H$_2$O$_2$ at 0-240 μM and Cl$^-$ at 140 mM. The MPO activity of the disrupted PMNs was determined during a 5-min incubation period at pH 7.4 and compared with the activity of purified human neutrophil MPO. A pH of 7.4 was used in the present study, since the pH within the phagolysosome has recently been reported to be 7.4-7.8 during the first 5-15 min following ingestion of opsonized zymosan particles (Cech and Lehrer, 1984; Segal et al., 1981). An incubation of 5 min was chosen as the appropriate reaction time based on the very rapid bactericidal activity of the intact PMN following bacterial ingestion (Cech and Lehrer, 1984).

We found that the activity of detergent disrupted PMNs was approximately equivalent to 0.1 unit of purified MPO (based on the specific activity of the purchased MPO) under the present assay conditions.

To determine $O_2(\Delta \lambda)$ production, DPA- and perylene-coated beads were added to 0.1 unit of purified MPO in PBS-D$_2$O, PBS-H$_2$O, and PBS with 75% D$_2$O and 25% H$_2$O all containing 140 mM Cl$^-$ and 0-90 μM H$_2$O$_2$. H$_2$O in the PBS was replaced with D$_2$O to prevent the rapid quenching of $O_2(\Delta \lambda)$ by H$_2$O (Merkel and Kearns, 1972). The MPO activity curve was similar in the various buffers, although detectable product formation was less during the 5 min of measurement in PBS-D$_2$O as compared with PBS-H$_2$O (Table II). The amount of $O_2(\Delta \lambda)$ generated during a 5-min incubation at 37°C by 0.1 unit of purified MPO in the various PBS buffer systems is presented in Table III. These values indicate that a large proportion of the unreacted H$_2$O$_2$ and HOCl generated by the MPO-catalyzed reaction subsequently react to form $O_2(\Delta \lambda)$ under the conditions used in this study.

The production of $O_2(\Delta \lambda)$ by the purified MPO system was completely inhibited by the addition of 1 mM histidine, a known water-soluble quencher of $O_2(\Delta \lambda)$ (K., $k_2 = 2.0 \times 10^8$ M$^{-1}$ s$^{-1}$) (Hodgson and Fridovich, 1974; Corey et al., 1987) (Table III). The addition of catalase, which catalyzes the conversion of H$_2$O$_2$ to water and ground state molecular oxygen, decreased the production of $O_2(\Delta \lambda)$ by this enzyme system in a concentration-dependent manner (Table III). In addition, warming the reacted samples to 120°C, as previously described, resulted in an 77 ± 3.0% dissociation of the DPA-endoperoxide in samples containing purified MPO and 30-90 μM H$_2$O$_2$.

### Table II

<table>
<thead>
<tr>
<th>H$_2$O$_2$</th>
<th>o-Dianisidine product in PBS containing % D$_2$O to H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>nmol</td>
</tr>
<tr>
<td>75%</td>
<td>nmol</td>
</tr>
<tr>
<td>0 μM</td>
<td>0 (3)</td>
</tr>
<tr>
<td>30 μM</td>
<td>8.1 ± 0.4 (3)</td>
</tr>
<tr>
<td>60 μM</td>
<td>12.7 ± 0.7 (3)</td>
</tr>
<tr>
<td>90 μM</td>
<td>16.1 ± 0.8 (3)</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>H$_2$O$_2$</th>
<th>O$_2(\Delta \lambda)$ quencher</th>
<th>Depletion of H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>nmol</td>
<td>nmol</td>
</tr>
<tr>
<td>75%</td>
<td>nmol</td>
<td>nmol</td>
</tr>
<tr>
<td>0 μM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 μM</td>
<td>3.8 ± 0.9 (4)</td>
<td>4.6 ± 0.7 (3)</td>
</tr>
<tr>
<td>60 μM</td>
<td>3.7 ± 0.6 (4)</td>
<td>3.6 ± 0.9 (3)</td>
</tr>
<tr>
<td>90 μM</td>
<td>2.7 ± 0.5 (3)</td>
<td>2.6 ± 1.9 (3)</td>
</tr>
<tr>
<td>$O_2(\Delta \lambda)$ quencher</td>
<td>50 μM + 1.0 mM histidine</td>
<td>0.0 ± 0.02 (3)</td>
</tr>
<tr>
<td>Depletion of H$_2$O$_2$</td>
<td>50 μM + 20 μg of catalase</td>
<td>1.4 ± 0.70 (2)</td>
</tr>
<tr>
<td></td>
<td>50 μM + 60 μg of catalase</td>
<td>0.0 ± 0.03 (2)</td>
</tr>
</tbody>
</table>
To further confirm the specificity of this method to detect \( O_2(\Delta \alpha) \) production by the MPO/H,O,\( /Cl^- \) system, NaOCl was added directly to the coated bead assay to determine if this product would react with the DPA- and perylene-coated beads. Although HOCl/OCl\( - \) does not react directly with DPA to give an endoperoxide, it might interfere with the 355 nm absorbance by forming a chlorinated product of DPA. The direct addition of 30–90 \( \mu M \) NaOCl did not result in a decreased absorbance at 355 nm or other wavelengths, confirming the nonreactivity of DPA with this molecule at pH 7.4.

**DISCUSSION**

In the present study, we present a specific and sensitive method for the intracellular detection of \( O_2(\Delta \alpha) \) production within phagocytically stimulated PMNs. To quantitatively detect \( O_2(\Delta \alpha) \) generation within PMNs, we have taken advantage of the specificity with which DPA reacts with \( O_2(\Delta \alpha) \) to form a stable and quantifiable product, DPA-endoperoxide. The development of this method relied on earlier studies establishing the slow reactivity rate of perylene and the fast reactivity rate of DPA with \( O_2(\Delta \alpha) \) and studies that confirmed the reversibility of the DPA reaction with \( O_2(\Delta \alpha) \) by warming (Wasserman et al., 1972; Turro et al., 1981). Using DPA- and perylene-coated beads we were able to detect the intracellular production of \( O_2(\Delta \alpha) \) by stimulated and PMA-costimulated PMNs adhered to fibronectin-coated coverslips. The amount of \( O_2(\Delta \alpha) \) detected, however, may not represent all of the \( O_2(\Delta \alpha) \) produced by these cells, since an unknown amount of the \( O_2(\Delta \alpha) \) will react with cellular components or will be quenched by water within the phagolysosome before coming into contact with the DPA-coated beads. The amount of \( O_2(\Delta \alpha) \) generated is approximately 19 ± 5.0% of the oxygen consumed by 1.25 × 10^6 PMNs stimulated in suspension. Previous attempts to assess the production of \( O_2(\Delta \alpha) \) by traps and quenchers have been questioned based on the non-specificity of their reaction profiles (Takeyama et al., 1977; Held and Hurst, 1978; Harrison et al., 1978) or the slow reactivity rate of the trap used to investigate the production of \( O_2(\Delta \alpha) \) (Koo and Schuster, 1978). The most specific method available for the detection of \( O_2(\Delta \alpha) \) is by near-IR emission spectroscopy, however, similar to the findings of Kanofsky and others (Kanofsky et al., 1988), we have been unable to detect a \( O_2(\Delta \alpha) \) infrared emission at 1268 nm in suspensions of stimulated PMNs because of the low sensitivity of this method. In the present study, the amount of \( O_2(\Delta \alpha) \) detected was essentially 100-fold higher than the previously reported values obtained by Foote et al. (1981) of ≤2.0 ± 2.0%/10^6 cells or 0.25%/1.25 × 10^6 cells of the oxygen consumed using [\(^1^4\)C]cholesterol as a \( O_2(\Delta \alpha) \) trap. The reaction rate of \( O_2(\Delta \alpha) \) with DPA is also 100-fold greater than the reaction rate of cholesterol and \( O_2(\Delta \alpha) \).

The involvement of \( O_2(\Delta \alpha) \) in the respiratory burst of PMNs was suggested some time ago, based on the emission of light by PMNs during phagocytosis (Allen et al., 1972; Allen, 1975) and given the fact that bacteria rich in carotenoid pigments resist killing by PMNs (Krinsky, 1974). Allen et al. (1972) proposed that the MPO/H,O,\( /Cl^- \) system might be a source of \( O_2(\Delta \alpha) \) generation in phagocytically stimulated PMNs. This suggestion was based on the early work of Khan and Kashra (1963) that established the production of \( O_2(\Delta \alpha) \) in a chemical reaction between H,O, and HOCl (Khan and Kashra, 1970). It has been suspected previously that the poor yield of \( O_2(\Delta \alpha) \) by this system could be the result of product inactivation of the MPO (Kanofsky et al., 1984). It has only been reported recently that quantities of the substrate H,O, above 100 \( \mu M \) can also lead to the inactivation of MPO at pH 7.5 and that the physiological concentration of H,O, in the phagolysosome is probably less than 100 \( \mu M \) (Kettle and Winterbourn, 1988; Zaubier et al., 1990). Therefore, detection of \( O_2(\Delta \alpha) \) production by this enzyme system has been unsuccessful because nonphysiological concentrations of H,O, ≥250 \( \mu M \) have routinely been used with physiological amounts of MPO. We were able to demonstrate in the present study, using DPA- and perylene-coated beads, that at concentrations of H,O, of 30–60 \( \mu M \) the MPO/H,O,\( /Cl^- \) system generated significant amounts of \( O_2(\Delta \alpha) \) and that the production of \( O_2(\Delta \alpha) \) decreased with increasing concentrations of H,O,.

The reaction rate of DPA with \( O_2(\Delta \alpha) \) is 100-fold greater than the reaction rate of cholesterol at pH 7.4. The efficiency of the present MPO system to generate \( O_2(\Delta \alpha) \) was calculated to be 43% at 30 \( \mu M \) H,O, with the efficiency dropping to 19% at 90 \( \mu M \) H,O,. suggesting that the MPO/H,O,\( /Cl^- \) system may serve as a source of \( O_2(\Delta \alpha) \) under physiological conditions in the intact PMN and play a role in the enhanced bactericidal activity of PMNs that contain this enzyme.

The amounts of \( O_2(\Delta \alpha) \) generated by 0.1 unit of MPO/ H,O,\( /Cl^- \) system did not completely equal the amounts of \( O_2(\Delta \alpha) \) detected in the intact PMN. Although the assay conditions including the aqueous environment and the amount of enzyme used may in part explain the apparent discrepancy in these values, another possibility would be that a second source of \( O_2(\Delta \alpha) \) exists in these cells. Superoxide anion is known to undergo spontaneous dismutation to H,O, and O, in the presence of hydrogen ions. Allen (1975) postulated that the O, produced during dismutation could be in the singlet excited state (\( \Delta \alpha \)). The generation of H,O, and \( O_2(\Delta \alpha) \) by the direct interaction of two molecules of O, is summarized in Reaction 3.

\[
O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2(\Delta \alpha)
\]

The suggestion by Allen was based on a proposal by Khan (1970) that an electron transfer reaction between two molecules of O, could lead to the formation of electronically excited \( O_2(\Delta \alpha) \). It was later verified that \( O_2(\Delta \alpha) \) was produced in a H,O,-induced dismutation reaction (Khan, 1981; Corey et al., 1987). It is therefore possible that the dismutation of O, produced by the phagocytically stimulated PMNs plays a direct role in the production of \( O_2(\Delta \alpha) \). At the same time, production of large amounts of O, would limit the amount of \( O_2(\Delta \alpha) \), since it is an efficient quencher of \( O_2(\Delta \alpha) \) when present at high concentrations (Khan, 1978).

In summary, we have developed a specific and sensitive method to measure the intracellular production of \( O_2(\Delta \alpha) \) have and demonstrated that PMNs generate \( O_2(\Delta \alpha) \). Our findings suggest that the MPO/H,O,\( /Cl^- \) system is a source of the \( O_2(\Delta \alpha) \) produced during the process of phagocytosis. A role for \( O_2(\Delta \alpha) \) in PMN-mediated damage of normal tissues in a variety of inflammatory disease states or its important role in mediating the destruction of infectious agents during host defense responses must now be considered.

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