The effect of the NADPH oxidase inhibitor diphenyleneiodonium on aerobic and anaerobic microbicidal activities of human neutrophils

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NADPH-dependent superoxide production by intact human neutrophils is inhibited by DPI (diphenyleneiodonium), when stimulated by either FMLP (*N*-formylmethionyl-leucyl-phenylalanine) or PMA (phorbol 12-myristate 13-acetate). Addition of 10 μ M-DPI abolished the reduction of both the FAD and the cytochrome *b* components of the NADPH oxidase. DPI inhibition of the oxidase was associated with defective aerobic killing of staphylococci by human neutrophils. Anaerobic killing, phagocytosis, chemotaxis and motility were relatively unaffected by 10 μ M-DPI. Degranulation of the azurophil and specific granules, induced by the soluble stimuli FMLP or PMA, and by particulate stimuli was decreased by the presence of DPI. The above effects of DPI on human neutrophils are similar to those found in chronic granulomatous disease.

INTRODUCTION

Neutrophils play an important role in killing infecting micro-organisms. During phagocytosis of bacteria, stimulation of neutrophil plasma-membrane NADPH oxidase leads to the production of reactive oxygen intermediates (see review by Rossi, 1986). The oxygen radicals produced and their subsequent reaction products are directly toxic to micro-organisms, and the NADPH oxidase may also be important in regulation of phagosome pH (Segal *et al.*, 1981; Henderson *et al.*, 1987), which may influence the activity of granule proteins discharged into the phagosome.

Neutrophils isolated from patients with chronic granulomatous disease (CGD) have commonly been used to determine which leucocyte functions are dependent on NADPH oxidase activity. CGD neutrophils lack the capacity to generate oxygen radicals, owing to defects in their NADPH oxidase, but, because of the low frequency of the disease (1 in 400 000), such cells are often difficult to obtain.

Previous studies in this laboratory have shown that diphenyleneiodonium (DPI) inhibits the NADPH oxidase of pig neutrophils (Cross & Jones, 1986; Cross, 1987) and rat peritoneal macrophage (Hancock & Jones, 1987). It binds covalently to a polypeptide (M_r 45 000) of the plasma membrane, a reaction diminished in the presence of NADPH. A peptide of similar M_r is phosphorylated when NADPH oxidase of neutrophils is activated (Gennaro *et al.*, 1985) and is absent, or altered, in neutrophils of patients with the autosomal recessive form of CGD (Segal *et al.*, 1985).

In the present study we describe the effects of DPI on the NADPH oxidase of human neutrophils and also on a variety of other human neutrophil functions, including aerobic and anaerobic bactericidal activity, degranulation, phagocytosis, chemotactic and random migration.

METHODS AND MATERIALS

Isolation of human neutrophils

Neutrophils were isolated from buffy coats (kindly supplied by South West Regional Blood Transfusion Centre, Southmead Hospital, Bristol, U.K.; used 16 h after collection) by dextran sedimentation and centrifugation through Ficoll (Histopaque-1077; Sigma); erythrocytes were removed by two cycles of haemolysis in 0.2% (w/v) NaCl containing 1 mM-EDTA and 5 μ g of heparin/ml, followed by restoration of iso-osmoticity by addition of an equal volume of 1.6% NaCl (modified from Segal & Darling, 1980). Cells were finally suspended in modified Krebs–Ringer (Cross *et al.*, 1984) buffer, pH 7.4, at approx. 5×10^7 cells/ml.

Determination of $O_2^{\cdot -}$ production

Neutrophils were suspended in a cuvette containing 1 ml of 100 μ M horse heart cytochrome *c* (Sigma, type III) in Krebs–Ringer buffer, pH 7.4, at 37 °C, and a stimulus of the oxidase was added. The rate of superoxide dismutase-inhibitable cytochrome *c* reduction was then recorded at 550 nm minus 540 nm (Cross *et al.*, 1982).

Bactericidal assays

The Oxford strain of *Staphylococcus aureus* (N.C.T.C. 6571) was used in bactericidal assays. Bacteria were prepared and bactericidal assays performed as described by Williams *et al.* (1985), with human in place of bovine neutrophils. Lysostaphin was used to remove extracellular staphylococci in the determination of intracellular survival (Tan *et al.*, 1971).

Neutrophils (3.3×10^6 /ml) and bacteria (10^6 /ml) were suspended in 20 mM-phosphate-buffered saline (0.137 M-NaCl/2.68 mM-KCl/8.1 mM- Na_2HPO_4 /1.47 mM- KH_2PO_4 , pH 7.0) containing 1.26 mM- $CaCl_2$, 0.41 mM-

Abbreviations used: DPI, diphenyleneiodonium; PMA, phorbol 12-myristate 13-acetate; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; CGD, chronic granulomatous disease.

MgSO₄, 0.5 mM-MgCl₂, 5.5 mM-glucose and 10% (v/v) human serum previously heated to 56 °C for 30 min.

For anaerobic assays, phosphate-buffered saline was deoxygenated by bubbling with O₂-free N₂ for 2 h. Suspensions of bacteria, neutrophils and the final assay mixtures were then prepared in an anaerobic cabinet (dw Scientific) with an atmosphere of N₂/H₂/CO₂ (8:1:1). All assays were performed in 2 ml Vacutainer tubes (BDH) opened in the appropriate atmosphere. These tubes have gas-tight rubber stoppers which allow the addition or subtraction of liquids by needle and syringe without introduction of air.

Assay mixtures were incubated with rolling for 90 min at 37 °C.

Bacterial counting

Counts of viable bacteria were made by drop-count method (Miles *et al.*, 1938).

Total and intracellular survival and phagocytosis were calculated as described by Williams *et al.* (1985). Total survival is expressed as the percentage of viable bacteria present at the end of the incubation, phagocytosis as the percentage of bacteria ingested by neutrophils (allowing for those adherent to neutrophils but not internalized), and intracellular survival as the percentage of those bacteria actually phagocytosed which survive intracellularly. This estimate of phagocytosis assumes that bacteria have to be phagocytosed to be killed.

Chemotactic migration

Chemotactic migration of neutrophils under agarose was measured by a modification of the technique of Nelson *et al.* (1975). In this, 7.5 ml of 1% agarose in Hanks balanced salt solution containing 0.2% human serum albumin and buffered with 20.0 mM-Hepes, pH 6.8, was dispensed into a 90 mm-diameter plastic Petri dish (Sterilin). With a cutter and template kit (Miles Laboratories), rows of three holes, 5 mm in diameter and 3 mm apart, were cut in the agar. The centre well was filled with 30 µl of chemoattractant, and the outer wells were filled with 30 µl of a suspension of neutrophils (10⁷ cells/ml) in Hanks balanced salts solution. Chemoattractants used were fresh human serum and 0.1 µM-FMLP (Sigma). Plates were incubated in a humid atmosphere for 3 h at 37 °C and then flooded with methanol for 20 min to fix the cells; the agar was carefully removed, and plates were stained with Leishmann's stain.

Neutrophil migration was quantified by measuring the distance that cells had moved from the edge of the well towards the chemoattractant, by means of microscopic observation and the use of a 10 × 10 square graticule. The leading edge of the migrating neutrophils was defined as the last graticule unit beyond which at least six squares were devoid of neutrophils for at least two rows (Repo, 1977).

Random migration

Random-migration plates contained 5% (v/v) heated (56 °C; 30 min) human serum instead of human serum albumin, and individual wells (5 mm diameter) were cut at random positions in the agar. After incubation and fixation, as for chemotactic migration, four measurements at right angles were taken from each well to the outer edge of neutrophil migration.

Fig. 1. Effect of DPI on superoxide production by human neutrophils induced by PMA or FMLP

Intact neutrophils ($\sim 5 \times 10^6$ /ml) were suspended, at 37 °C, in Krebs-Ringer buffer, pH 7.4, containing 100 µM-cytochrome *c*. Stimuli were FMLP (10 µM; ■) or PMA (17 nM; ▲), and concentrations were adjusted to give equal rates of O₂⁻ production in the absence of DPI. O₂⁻ was measured as the superoxide-inhibitable rate of cytochrome *c* reduction (Cross *et al.*, 1982).

DPI

DPI (kindly given by Dr. A. R. Cross of this Department) was included in bactericidal, chemotactic- and random-migration assays at a final concentration of 10 µM. Controls contained equivalent volumes of its solvent, 95% ethanol.

Statistical analysis

The values of intracellular and total survival and phagocytosis were compared by analyses of variance in a model which included terms for assay, environment (aerobic or anaerobic) and the presence or absence of DPI. The model was fitted in stages to the logarithm of the variate by using the general least-squares method.

A similar model and method were used to compare distances migrated towards chemoattractant or distances moved in random migration. Terms were included for assay, chemoattractant and drug.

Determination of degranulation

Neutrophils (5×10^7 /ml) in Krebs-Ringer buffer, pH 7.4, were prewarmed to 37 °C in 500 µl portions in a shaking water bath. Degranulation was initiated by addition of FMLP (10 µM), or opsonized zymosan or opsonized latex beads (25.7 µm diam.; to promote 'frustrated phagocytosis'), or PMA (250 nM); with FMLP or particulate stimuli, the cells were pre-treated for 10 min with 3 µg of cytochalasin B. After 30 min incubation the suspensions were chilled at 0 °C and centrifuged. Supernatants were assayed for vitamin B₁₂-binding protein (Kane *et al.*, 1974) and myeloperoxidase (Baggiolini, 1974). Techniques for opsonizing particles and their use in degranulation assays were as described by Markert *et al.* (1984) and Segal & Jones (1979).

Fig. 2. Effect of DPI on steady-state absorption spectra of human neutrophils

Neutrophils ($\sim 5 \times 10^7$ /ml) were suspended in phosphate-buffered saline, pH 7.4, containing glucose (5 mM), in reference and sample cuvettes of a sensitive spectrophotometer. A baseline was recorded; then PMA (250 nM) was added to the sample cuvette and a spectrum recorded. DPI (10 μ M) was then added to the sample and a spectrum recorded. Sodium dithionite (a few crystals) was finally added to give complete reduction of the membrane cytochrome *b*. Traces are difference spectra: (a) +PMA minus baseline; (b) +PMA+DPI minus baseline; (c) +sodium dithionite minus baseline.

Spectroscopy

Absorption spectra were recorded with a scanning split-beam spectrophotometer with facilities for averaging spectra (Cross *et al.*, 1985).

Other assays

Lactate dehydrogenase was assayed as described by Neilands (1955). Trypan Blue exclusion was assayed as described by Metcalf *et al.* (1986).

RESULTS

Effect of DPI on NADPH oxidase

When DPI was added to human neutrophils, their capacity for $O_2^{\cdot -}$ generation on addition of FMLP or PMA was severely decreased (Fig. 1). The concentrations of the stimuli were adjusted to give equal rates of uninhibited $O_2^{\cdot -}$ production, but the PMA response was consistently more sensitive to DPI than was the FMLP response.

When DPI was added to intact human neutrophils, effects were observed which indicated that DPI was acting on an electron-transport system containing cytochrome *b* and, probably, flavin. Treatment of aerobic neutrophils with PMA (Fig. 2) caused an increase in absorbance at 429 nm, corresponding to the γ -band of cytochrome *b*-245, together with a trough at about 450 nm, corresponding to the reduction of FAD (Cross *et al.*, 1985). Both changes were abolished when 10 μ M-DPI was added to the stimulated cells. The addition of sodium dithionite to the sample cuvette increased the

Table 1. Effect of DPI on aerobic and anaerobic killing of *S. aureus* by human neutrophils

The assays were described in the Methods and materials section. Results are averages of three experiments; * result significantly different ($P < 0.05$) from same parameter measured in aerobic conditions without DPI.

	Aerobic incubation		Anaerobic incubation	
	-DPI	+DPI (10 μ M)	-DPI	+DPI (10 μ M)
Total survival (%)	37	69*	59*	50*
Intracellular survival (%)	20	50*	42*	47*
Phagocytosis (%)	77	76	71	89

reduction of the cytochrome *b* and the reduction of myeloperoxidase, which absorbs at 475 nm.

Effect of DPI on bactericidal functions of human neutrophils

The addition of DPI greatly diminished the killing of *S. aureus* by human neutrophils in aerobic conditions (Table 1). Both total and intracellular survival of bacteria were increased ($P < 0.05$) when DPI was present. In anaerobic conditions total and intracellular survival were unaffected by DPI, although killing was significantly decreased ($P < 0.05$) in the absence of O_2 . This experiment indicates that aerobic killing processes are dependent on NADPH oxidase activity of neutrophils; when this is abolished by DPI, bacterial killing is less efficient and is similar to that found in anaerobic conditions. Phagocytosis was unaffected by DPI or by anaerobiosis.

Neutrophil migration

DPI (10 μ M) caused a small but significant depression of the chemotactic response of neutrophils to FMLP by 9.5% ($P < 0.05$) and to serum by 9.2% ($P < 0.001$).

DPI had no effect on random migration.

Neutrophil degranulation

The release of both myeloperoxidase (an azurophil-granule marker) and vitamin B_{12} -binding protein (a specific-granule marker) was inhibited by the addition of DPI when the soluble stimuli FMLP or PMA were used (Table 2; Fig. 3). DPI had no effect on the assays for the released myeloperoxidase or for vitamin B_{12} -binding protein.

In experiments using particulate stimuli of degranulation, it was found that 10 μ M-DPI did not inhibit the release of myeloperoxidase stimulated by opsonized zymosan, and inhibited myeloperoxidase release stimulated by opsonized latex beads from 45% to 33% of the total enzyme. DPI inhibited release of vitamin B_{12} -binding protein, induced by the same stimuli, from 64% to 52% and 45% to 33% of the total protein respectively.

Cytotoxicity of DPI

Neutrophils exposed to 10 μ M-DPI were tested for any cytotoxic effects of this compound. There was no effect on Trypan Blue exclusion (> 97% after 30 min) or on lactate dehydrogenase release; both untreated neutro-

Fig. 3. Effect of DPI on degranulation of human neutrophils induced by addition of PMA or FMLP

Assay conditions were as described in Table 2. Vitamin B₁₂-binding protein release induced by PMA (▲) or FMLP (■); myeloperoxidase release induced by PMA (△) or FMLP (□).

phils and DPI-treated neutrophils released 5–10% of lactate dehydrogenase over this period. Microscopic examination of the phagocytosis of fluorescent-labelled *S. aureus* showed that DPI was without effect.

DISCUSSION

In whole human neutrophils, DPI clearly abolished the cytochrome *b* reduction which resulted from activation of the NADPH oxidase by PMA (Fig. 2), and there was, but less clearly seen, an increase in absorbance at around 450 nm, presumed to be due to oxidation of reduced flavin. (Spectroscopic changes in whole cells are complicated by the presence of myeloperoxidase, with a strong absorbance at 475 nm, which is altered on activation of the cells.) It therefore seems likely that the site of action of DPI is the same as that found for the solubilized NADPH oxidase of pig neutrophils (Cross & Jones, 1986), where it was proposed to act on, or before, the flavoprotein in an electron-transport system which had the sequence: NADPH → FAD protein → cytochrome *b* → O₂.

In X-linked CGD, the failure of neutrophils to produce O₂^{•-} in response to stimulation is associated with the absence of the low-potential cytochrome *b* component of the NADPH oxidase (Segal *et al.*, 1983). This has been shown to arise from a defect of the gene coding for the β-subunit of cytochrome *b* (Tehan *et al.*, 1987). Patients with CGD suffer recurrent severe infections, particularly by staphylococci and enteric bacteria, as well as by the fungi *Candida albicans* and *Aspergillus* sp. These organisms are poorly killed by isolated CGD neutrophils, but other bacteria, such as streptococci and *Lactobacillus acidophilus*, are killed well (see review by Babior & Crowley, 1983).

Table 2. Effect of DPI on degranulation induced in human neutrophils by the soluble stimuli FMLP and PMA

Conditions for degranulation assays were described in the Methods and materials section. Where present, DPI (10 μM) was added 10 min before the stimulus, and remained present for the duration of the incubation. Myeloperoxidase was used as a marker for azurophil granules, and vitamin B₁₂-binding protein for specific granules. Values quoted represent the proportion of total granule enzyme (supernatant from sonicated cells) that is released. Results are means ± S.E.M. for five experiments. Cell viability, tested by Trypan Blue exclusion and lactate dehydrogenase release, was unaffected by DPI treatment (see the Methods and materials section). Abbreviation: Cyt. B, is cytochalasin B.

Incubation conditions	Myeloperoxidase released (% of total)	Vitamin B ₁₂ -binding protein released (% of total)
No addition	11.3 ± 0.29	33.4 ± 1.28
+ PMA	64.8 ± 2.28	72.0 ± 3.26
+ PMA + DPI (10 μM)	43.0 ± 10.77	56.7 ± 13.94
+ FMLP + Cyt. B	30.4 ± 2.20	57.0 ± 2.94
+ FMLP + Cyt. B + DPI (10 μM)	17.3 ± 1.88	43.0 ± 0.85
+ Cyt. B	13.0 ± 0.45	40.5 ± 0.83
+ Cyt. B + DPI (10 μM)	13.4 ± 0.57	32.1 ± 0.33
+ DPI (10 μM)	13.4 ± 0.41	29.2 ± 1.02

The aerobic killing of *S. aureus* by neutrophils is diminished by DPI treatment to values similar to those seen in anaerobic conditions (Table 1), where the very low O₂ concentration prevents an effective respiratory burst and bactericidal activity is decreased (Mandell, 1974). DPI-treated neutrophils behave much like CGD neutrophils in this respect: neither can generate O₂^{•-} in response to phagocytic stimuli, and this severely affects their microbicidal function. In CGD neutrophils non-oxygen-dependent killing can still proceed, and this indicates that chemotaxis, migration, phagocytosis and degranulation to release microbicidal proteins still occurs (Babior & Crowley, 1983). We have found that the migration of human neutrophils is almost unaffected by DPI treatment, and that phagocytosis too is very little affected. These are complex membrane receptor-initiated events which require the integration of many enzyme systems which are obviously resistant to inhibition by 10 μM-DPI. In contrast, we have found that neutrophil degranulation is decreased to some extent by DPI (Table 2; Fig. 3), when soluble or particulate stimuli are used. This may be a direct effect of DPI on the cytoskeletal changes necessary for degranulation, or it might be a consequence of the inhibition of NADPH oxidase by DPI. This oxidase is electrogenic, and its activity leads to persistent depolarization of the plasma membrane (Henderson *et al.*, 1987) and to intracellular changes in pH; the membrane depolarization is likely to affect voltage-regulated ion channels in the plasma membrane. If such changes are necessary for efficient fusion of the granules with the inner face of the plasma membrane

during degranulation, the inhibition of NADPH oxidase by DPI might affect this process. There are reports (Babior & Crowley, 1983; Gold *et al.*, 1974) that in CGD neutrophils degranulation is slower and diminished in extent, although phagocytosis is normal (Segal *et al.*, 1982).

It is noteworthy that even though degranulation is partly inhibited by DPI, this does not affect the anaerobic killing reactions (Table 1), which depend on degranulation.

Our results lead us to suggest that normal human neutrophils treated with DPI may be a valuable alternative to the poorly available CGD neutrophils in examining the contribution of NADPH oxidase-derived activated oxygen species to a variety of leucocyte functions, including cytotoxic activities. We and our colleagues have made use of this inhibitor in the study of intracellular and intraphagosome pH changes following neutrophil activation and in examining the relative contribution of oxygen radicals to the tumoricidal activity of phagocytes.

This work has been supported by grants from the Medical Research Council; S.J.M. is supported by an A.F.R.C. Veterinary Fellowship and J.A.E. by an S.E.R.C. Studentship.

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Received 24 June 1987/12 October 1987; accepted 7 January 1988