JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE VOL 6 29–34 (1991)

Effect of Different Inhibitors on the Intracellularly and Extracellularly Generated Chemiluminescence Induced by Formylmethionyl-leucyl-phenylalanine in Polymorphonuclear Leukocytes. Cellular Response in the Presence of Mannitol, Benzoate, Taurine, Indomethacin and NDGA

Claes Dahlgren
Department of Medical Microbiology, Linköping University, S-581 85 Linköping, Sweden

When polymorphonuclear leukocytes (PMNL) interact with the soluble stimulus formylmethionyl-leucyl-phenylalanine (FMLP), the cells increase their production of oxidative metabolites. This increased production can be measured as lumino-amplified light emission or chemiluminescence (CL). In the present report, experimental systems which allow a quantitation of extracellularly and intracellularly generated metabolites have been used, and the effect of mannitol, benzoate, taurine, indomethacin and nordihydroguaiaretic acid has been investigated. The presence of the hypochlorous acid scavenger taurine had no effect on the intracellular response, whereas the extracellular response was reduced with around 50%. The hydroxyl radical scavenger mannitol had only minor effects on the response, whereas benzoate, another hydroxyl radical scavenger, reduced the extracellular response with around 50% and the intracellular response with more than 90%. Indomethacin, an inhibitor of arachidonic acid metabolism, did not influence the response, whereas NDGA, also an inhibitor of the arachidonic acid metabolism, totally abolished both the extracellular and the intracellular response. The use of scavengers/inhibitors as a means of determining the mechanisms of light emission, and the origin of chemiluminescence produced by neutrophils stimulated by FMLP is discussed.

Keywords: Chemiluminescence; luminol; scavengers; human neutrophils

0884-3996/91/010029-06$05.00

Received January 1990

INTRODUCTION

When polymorphonuclear leukocytes (PMNL) interact with soluble or particulate matter, the cells respond with a burst in oxidative metabolism generating chemically reactive molecules, e.g. superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2). This activation is an essential step in host defence against invading microorganisms (Klebanoff and Clark, 1978). Since the techniques used to quantitate superoxide and hydrogen peroxide usually involve some large molecular detector molecule that cannot obtain access to intracellular sites (Metcalf *et al.*, 1986), it is generally accepted that the O_2^-/H_2O_2 generating system is localized in the plasma membrane of PMNL, and that the produced radicals are released extracellularly. Since extracellularly generated/produced metabolites have been shown to be of importance in tissue destruction (Movat, 1985), as well as for different regulatory processes (Clark and Szot, 1982; Stendahl *et al.*, 1984) measurement of the released metabolites are of biological relevance. However, to minimize destruction of tissue components, and to make an effective contribution to the killing of invading microorganisms, the cellularly produced oxidative metabolites should be released inside a phagosome, indicating that measurement also of intracellularly generated metabolites is of importance.

The generation of oxygen metabolites is accompanied by light emission or chemiluminescence (CL) by the PMNL (Allen *et al.*, 1972; Allen and Loos, 1976). Addition of luminol to CL systems, has been shown by many investigators, to amplify the response, and results obtained using the luminol-dependent chemiluminescence technique show, that with this technique the generation of oxidative metabolites from intracellular events is also measured (Bender and vanEpps, 1983; Campbell and Hallett, 1983). However, to be able to quantify the contribution of intracellular and extracellular events in a cellular response, it must be possible to selectively inhibit the two reactions, i.e., to be able to measure chemiluminescence from an intracellular location, the extracellularly generated luminescence has to be reduced and vice versa. Chemiluminescence data obtained with cells from patients with peroxidase deficiencies have indicated that the luminol-dependent CL is dependent on peroxidase (Stevens *et al.*, 1978; DeChatelet *et al.*, 1982; Dahlgren and Stendahl, 1983), and HOCL produced by the MPO-H_2O_2-Cl reaction has been proposed to be responsible for the oxidation and light emission from luminol (DeChatelet *et al.*, 1982; Brestel, 1985). Thus, catalase, a large molecular hydrogen peroxide scavenger that cannot readily gain access to intracellular sites of PMNL, is a suitable inhibitor for extracellular chemiluminescence (Dahlgren, 1987a), and a system with azide, an MPO inhibitor, combined with an azide-insensitive peroxidase, is a suitable system for measurement of extracellularly produced luminescence (Wymann *et al.*, 1987; Dahlgren, 1988a). The precise mechanisms for the generation of light in these two systems are not known, but usually different scavengers of oxidative metabolites and different enzyme inhibitors are used to determine the relative importance of different reactions (Rosen and Klebanoff, 1976; Williams and Cole, 1981; Dahlgren, 1987a). The present investigation was thus performed to study the sensitivity for different inhibitors of intracellular and extracellular reactions, respectively, induced in human PMNL as a result of an interaction with the chemoattractant FMLP.

MATERIALS AND METHODS

Cells

To obtain normal granulocytes (polymorphonuclear leukocytes; PMNL), heparinized blood from apparently healthy adult volunteers was separated by the method of Bøyum (1968). After dextran sedimentation, hypnotic lysis and Hypaque-Ficoll centrifugation, the PMNL were washed (×2), counted with a Coulter Counter ZF (Coulter Electronics Ltd) and resuspended to 2×10^7/ml in Krebs-Ringer phosphate buffer, supplemented with glucose (10 mmol/l), Ca^{2+} (1 mmol/l) and Mg^{2+} (1.5 mmol/l) (KRG, pH 7.3). In order to allow surface exposure of the FMLP receptors, the cells were conditioned at room temperature for 60 min (Dahlgren *et al.*, 1987), in polypropylene tubes to which the cells adhere poorly. The cells were then transferred to a melting ice bath.

Reagents

Formylmethionyl-leucyl-phenylalanine (FMLP), horseradish peroxidase (HRP), taurine, indomethacin, nordihydroguaiaretic acid (NDGA) and luminol were obtained from Sigma (Sigma Chemical

Co., St Louis, MO). Catalase was obtained from Boeringer Mannheim (FRG). Benzoate was obtained from Kockens AB (Gävle, Sweden) and mannitol from BDH Chemical Ltd (Poole, England). The scavengers/inhibitors were dissolved in KRG and used in concentrations (final concentrations are given in Table 1) that did not affect cell viability. Dimethyl sulphoxide was used to dilute FMLP to 10^{-2} mol/l and water containing NaOH (0.1 mol/l) was used to dissolve luminol to 2×10^{-2} mol/l. These substances were further diluted in KRG to concentrations that were without effect on pH (NaOH) or hydroxl radical production (DMSO; Ward *et al*, 1983).

Chemiluminescence measurements

Chemiluminescence (CL) was measured in a six-channel Biolumat LB 9505 (Berthold Co, Wildbad, FRG), using disposable 4-ml polypropylene tubes with a 1.0-ml reaction mixture (pH 7.3). Samples were withdrawn (0.05 ml to a final volume of 1 ml) from the cell suspension and used for chemiluminescence measurement. The tubes were placed in the Biolumat and allowed to equilibrate for 5 min at 37 °C with or without an inhibitor. To activate the system, 0.1 ml of FMLP was added, and the light emission recorded continuously. Two different reaction mixtures were used for quantitation of intracellularly and extracellularly generated luminescence, respectively. Tubes used for measurement of extracellularly generated CL contained PMNL (10^6), HRP (4 U), azide (1 mmol/l) and luminol (4×10^{-6} mol/l). Tubes used for measurement of intracellularly generated CL contained PMNL (10^6), catalase (2000 U) and luminol (2×10^{-5} mol/l).

RESULTS

FMLP-elicited chemiluminescence (CL) from neutrophils

When neutrophils were exposed to FMLP a bimodal CL response was obtained. The first peak appeared at around 1 min after addition of FMLP and the second peak of activity occurred 5–7 min after stimulus addition. When the cells were exposed to FMLP in the presence of azide and HRP, a one-peak response with a peak corresponding to the initial peak of the bimodal response was obtained, whereas in the presence of catalase the peak activity corresponded to the second peak of the native response. The extracellular and intracellular parts of the response, were thus quantified as the peak value of the 'first' peak (A in Fig. 1) in the presence of azide and HRP and the 'second' peak

Table 1. Extracellular and intracellular chemiluminescence obtained from normal PMNL responding to FMLP (10^{-7} mol/l) and the effect of different scavengers and inhibitors. The values presented without any inhibitor represent the mean value ± SD (CPM x 10^{-6}) of 29 determinations, and the results obtained with the scavengers/inhibitors are expressed as a percentage of the value obtained without inhibitor (numerals represent mean value ± SD of five determinations).

Inhibiting substance	Cellular chemiluminescence	
	Extracellularly	Intracellularly
None	116 ± 83	51 ± 24
Taurine (10 mmol/l)	40.5 ± 2.6	108.7 ± 16.9
Mannitol (25 mmol/l)	102.3 ± 28.0	96.2 ± 17.7
Na-benzoate (10 mmol/l)	49.0 ± 12.6	6.9 ± 3.6
Indomethacin (5 μmol/l)	101.2 ± 7.5	90.1 ± 36.2
NDGA (10 μmol/l)	1.6 ± 2.1	3.6 ± 4.4

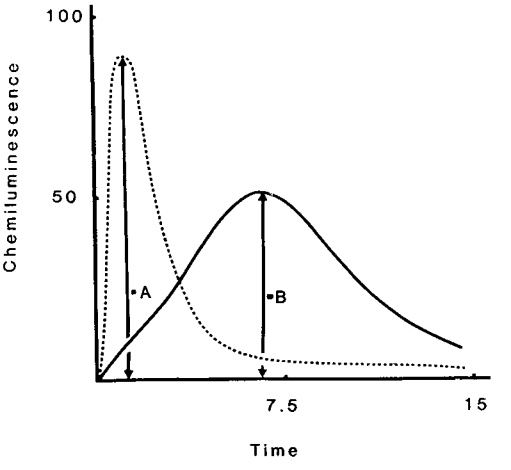

Figure 1. Time trace of extracellularly (dotted line) and intracellularly (solid line) generated chemiluminescence emitted from neutrophils when exposed to FMLP. The system used to measure extracellularly CL contained azide (1 mol/l) and HRP (4 U), and the system used to measure intracellular CL contained catalase (2000 U). The peak values A and B for the extracellular and intracellular response, respectively, were used to quantitate the two responses. Abscissa, time after addition of FMLP (minutes); ordinate, chemiluminescence in counts per minute ($\times 10^{-6}$).

(B in Fig. 1) in the presence of catalase, respectively. With the experimental set-up used, the extracellular response was twice as high as the intracellular response (Table 1).

Effect of taurine on extra and intracellular CL

Hypochlorous acid produced as a result of the peroxidase-H_2O_2-halide interaction has been proposed as being directly involved in the luminol-dependent CL. Taurine is a HOCl scavenger, and should thus inhibit the CL activity dependent on HOCl. From the CL measurements performed in the presence of taurine, it could be calculated, that more than 50% of the extracellular response disappeared, whereas the intracellular response was unaffected or even increased in the presence of taurine (Table 1).

Effect of hydroxyl radical scavengers on extra and intracellular CL

Hydroxyl radicals (OH·) is formed as an intermediate in secondary reactions involving hydrogen peroxide, superoxide and metal ions, and has also been proposed as a candidate for light emission. Two different OH· scavengers were used, and the effects of the two were quite different. Mannitol had only minor effects on the responses, whereas benzoate reduced the extracellular response with around 50% and the intracellular response with more than 90% (Table 1).

Effects of inhibitors of arachidonic acid metabolism

Inhibitors of arachidonic acid metabolism were used to study the possibility of neutrophil CL being dependent on the activity of the lipoxygenase and/or the cyclooxygenase pathways. NDGA was used to inhibit lipoxygenase and indomethacin to inhibit the cyclooxygenase pathway. Indomethacin had no effect on the extracellular response and only a small inhibitory effect on the intracellular response. NDGA totally inhibited both the intracellular and the extracellular response (Table 1).

DISCUSSION

Although luminol-amplified chemiluminescence (CL) has become increasingly popular for determining functional characteristics of PMNL, the mechanism(s) behind the reaction is far from understood (Trusch *et al.*, 1978; Klebanoff and Clark, 1978; Dahlgren, 1989). A number of publications dealing with the mechanism of light emission have shown that the CL reaction is dependent on cellular metabolism of molecular oxygen and on the granule enzyme myeloperoxidase (Aniansson *et al.*, 1984; Stevens *et al.*, 1978; DeChatelet *et al.*, 1982; Dahlgren and Stendahl, 1983; Dahlgren, 1988a). Furthermore, in contrast to many other techniques used to quantitate oxygen metabolites from granulocytes, much of the luminol-depdendent CL activity has been shown to have an intracellular origin (Dahlgren, 1987b; Dahlgren, 1988a; Bender and vanEpps, 1983; Stendahl *et al.*, 1984; Hallet and Campbell, 1983; Edwards, 1987). In order for a CL reaction to take place, the different constituents necessary for the reaction have to be present in the same subcellular compartment. For an extracellular reaction some of the oxygen metabolites produced are released to react with peroxidase in the extracellular milieu, whereas an intracellular reaction has to take place in a subcellular compartment without any contact with the surrounding environment. From these facts it could be concluded that the two systems used in this study, one containing catalase (a large molecular hydrogen peroxide scavenger) the other containing azide (an MPO inhibitor) combined with HRP (an azide insensitive peroxidase) allow us to quantitate intracellular and extracellular reactions, respectively (Dahlgren, 1988b, Dahlgren and Lock, 1988; Lock and Dahlgren, 1988). However, since both the intracellular and extracellular CL reaction is dependent on a number of different environmental factors such as ionic composition, pH, the presence of proteins and the luminol concentration (Briheim *et al.*, 1984; Dahlgren and Briheim, 1985), factors that affect the intracellular and extracellular milieu in different ways, no direct comparison in quantitative terms could be made.

One way to distinguish the relative importance of different oxidative metabolites in a measured cellular CL response, is to investigate the effects of different scavengers or specific inhibitors. However, since many of these substances are macromolecules (e.g. superoxide dismutase) that cannot gain access to intracellular sites and since the relative impor-

tance of the intracellular and extracellular part of the response varies depending on the stimulus used (Dahlgren, 1987b; Dahlgren and Lock, 1988), the lack of conclusiveness is obvious for many of the published reports dealing with effects of macromolecular scavengers. In this report, the inhibitory activity of a number of well-known scavengers/inhibitors has been investigated. These scavengers/inhibitors are small molecules or substances exerting their effect on the plasma membrane, and the results do illustrate some of the problems at hand, but do also shed some light on the importance of some of the candidates proposed as being involved in CL generation from phagocytes. The importance of the peroxidase in luminol-dependent CL has been established by several investigators, and HOCl produced has been proposed as being responsible for the oxidation and light emission from luminol. Taurine, that is a HOCl scavenger, is thus supposed to inhibit the response and, as can be seen in Table 1, the extracellular response is reduced by more than 50%. However, the intracellular response is not affected or even increased in the presence of taurine. The lack of conformity between the two systems could be due either to a difference in the light generating mechanism or to a restricted intracellular availability of the scavenger. Another candidate for CL generation is the hydroxyl radical (OH·), one of the most reactive biological compounds known, that is formed as an intermediate in reactions involving hydrogen peroxide, superoxide anions and metal ions. Benzoate is a well-known scavenger of OH·, and from Table 1 it is obvious that almost all intracellularly produced CL is removed by this scavenger. The extracellular response is reduced by only 50%, indicating differences in the mechanisms of the extracellular and intracellular response. Since almost all intracellular CL disappears, the difference observed between the two systems could not be due to a restricted availability of the scavenger. However, the role of OH· in the CL reactions could be questioned, since another OH· scavenger (mannitol) failed to inhibit the response independently of the localization of the CL activity. Indomethacin, an inhibitor of arachidonic acid metabolism, more specifically inhibiting the cyclooxygenase pathway, had no effect on the extracellular response, whereas the intracellular response was reduced by 10%. Indomethacin has been shown to have an inhibitory effect on MPO activity, indicating that the reduction of the intracellular CL could be due to the inhibitory effect on the MPO (Shacter *et al.*, 1987). The most potent inhibitor of both the intracellular and the extracellular reponse was NDGA, which almost eliminated the CL response in both the systems. NDGA is also an inhibitor of the arachidonic acid metabolism, however, the effect is on the lipoxygenase pathway, thus indicating a role for intermediates of the lipoxygenase pathway in the luminol-depedent CL. Whether this conclusion will hold true or if the effects measured are of an unspecific character just like the effects of some of the other scavengers, has to be further investigated.

In summary, this study shows that, despite the fact that measurement of luminol-dependent chemiluminescence could be a valuable and simple tool in studying metabolic activity in inflammatory cells, caution must be exercised when interpreting the cellular response to different agents since a number of different factors together determine magnitude, time-course and sensitivity to environmental factors of the response. Furthermore, since both intracellular and extracellular mechanisms seem to be involved in the cellular response, this may create problems in that the two reactions could be of variable importance in a response and that the mechanisms behind the two reactions seems to differ; but the CL systems should also provide an exciting area for future research on the importance of receptor activity in the pathogenesis of a number of disease processes.

Acknowledgements

The author acknowledges Kristina Orselius for excellent technical assistance. The work was supported by grants from the Swedish Medical Research Council, King Gustaf V 80-year foundation, The Swedish Association Against Rheumatism and the County of Östergötland.

REFERENCES

Allen, R. C. and Loos, L. D. (1976). Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophanges. *Biochem. Biophys. Res. Commun.*, **69**, 245-252.

Allen, R. C., Stjernholm, R. L. and Steel, R. H. (1972). Evidence for the generation of electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem. Biophys. Res. Commun.*, **47**, 679-684.

Aniansson, H., Stendahl, O. and Dahlgren, C. (1984). Comparison between luminol and lucigenin dependent chemiluminescence of polymorphonuclear leukocytes. *Acta. Path. Microbio. Immunol. Scand. Sect. C*, **92**, 357-361.

Bender, J. G. and vanEpps, D. E. (1983). Analysis of the bimodal chemiluminescence pattern stimulated in human neutrophils by chemotactic factors. *Infect. Immun.*, **41**, 1062-1070.

Bøyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1 × g. *Scand. J. Clin. Lab. Invest. Suppl.*, **97**, 77-89.

Brestel, E. (1985). Co-oxidation of luminol by hypochlorite and hydrogen peroxide implications for neutrophil chemiluminescence. *Biochem. Biophys. Res. Commun.*, **126**, 482-488.

Briheim, G., Stendahl, O. and Dahlgren, C. (1984). Intra-and extracellular events in luminol-dependent chemiluminescence of polymorphonuclear leukocytes. *Infect. Immun.*, **45**, 1-5.

Campbell, A. K. and Hallett, M. B. (1983). Measurement of intracellular calcium ions and oxygen radicals in polymorphonuclear leukocyte-erythrocyte 'ghost' hybrids. *J. Physiol.*, **338**, 537-550.

Clark, R. A. and Szot, S. (1982). Chemotactic factor inactivation by stimulated human neutrophils mediated by myeloperoxidase catalyzed methionine oxidation. *J. Immunol*, **128**, 1507-1513.

Dahlgren, C. (1987a). Polymorphonuclear leukocyte chemiluminescence induced by formylmethionyl-leucyl-phenylalanine and phorbol myristate acetate. Effects of catalase and superoxide dismutase. *Agents. Actions.*, **21**, 104-112.

Dahlgren, C. (1987b). Difference in extracellular radical release after chemotactic factor and calcium ionophore activation of the oxygen radical generating system in human neutrophils. *Biochem. Biophys Acta*, **930**, 33-38.

Dahlgren, C. (1988a). Analysis of luminol-dependent chemiluminescence from granule depleted neutrophil cytoplasts reveals two different light emitting mechanisms. *J. Biolum. Chemilum.*, **2**, 25-33.

Dahlgren, C. (1988b). Effects on extra- and intracellularly localized, chemoattractant induced, oxygen radical production in neutrophils following modulation of the conditions for ligand-receptor interaction. *Inflammation*, **12**, 335-349.

Dahlgren, C. (1989). Is lysosomal fusion required for the granulocyte chemiluminescence reaction? *Free Rad. Biol. Med.*, **6**, 399-403.

Dahlgren, C. and Briheim, G. (1985). Comparison between the luminol-dependent chemiluminescence of polymorphonuclear leukocytes and of the myeloperoxidase-HOOH system: influence of pH, cations and protein. *Photochem. Photobiol.*, **41**, 605-610.

Dahlgren, C. and Lock, R. (1988). The limitation of the human neutrophil chemiluminescence response by extracellular peroxidase is stimulus dependent: effect of added horse radish peroxidase on the response induced by both soluble and particulate stimuli. *J. Clin. Lab. Immunol.*, **26**, 49-53.

Dahlgren, C. and Stendahl, O. (1983). Role of myeloperoxidase in luminol dependent chemiluminescence of polymorphonuclear leukocytes. *Infect. Immun.*, **39**, 736-741.

Dahlgren, C., Andersson, T. and Stendahl, O. (1987). Chemotactic factor binding and functional capacity: a comparison between human granulocytes and differentiated HL-60 cells. *J. Leuk. Biol.*, **41**, 245-252.

DeChatelet, L. R., Long, G. D., Shirley, P. S., Bass, D. A., Thomas, M. L., Henderson, F. W. and Cohen, M. S. (1982). Mechanism of the luminol-dependent chemiluminescence of human neutrophils. *J. Immunol.*, **129**, 1589-1593.

Edwards, S. W. (1987). Luminol- and lucigen-dependent chemiluminescence of neutrophils:role of degranulation. *J. Clin. Lab. Immunol.*, **22**, 35-39.

Hallett, M. B. and Campbell, A. K. (1983). Two distinct mechanisms for stimulation of oxygen-radical production by polymorphonuclear leukocytes. *Biochem. J.*, **216**, 459-465.

Klebanoff, S. J. and Clark, R. A. (1978). *The Neutrophil: Function and Clinical Disorders*, Elsevier/North-Holland Biomedical Press, Amsterdam.

Lock, R. and Dahlgren, C. (1988). Characteristics of the granulocyte chemiluminescence reaction following an interaction between human neutrophils and *Salmonella typhimurium* bacteria. *APMIS*, **96**, 299-305.

Metcalf, J. A., Gallin, J. I., Nausseef, W. M. and Root, R. K. (1986). *Laboratory Manual of Neutrophil Function*, Raven Press, New York.

Movat, H. Z.. (1985). *The Inflammatory Reaction*, Elsevier, Amsterdam.

Rosen, H. and Klebanoff, S. J. (1976). Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. *J. Clin. Invest*, **58**, 50-60.

Shacter, E., Pati, S. and M. Potter, M. (1987). Inhibition by indomethacin of myeloperoxidase activity in pristane-elicited neutrophils. *J. Leuk. Biol.*, **42**, 430.

Stendhal, O., Andersson, T., Dahlgren, C. and Magnusson, K. -E. (1986). Defective chemiluminescence response in differentiated HL-60 cells due to impaired degranulation. *Biochem. Biophysis. Acta*, **881**, 430-436.

Stendhal, O., Coble, B. I., Dahlgren, C. and Hed, J. (1984). Myeloperoxidase modulates the phagocytic activity of polymorphonuclear neutrophil leukocytes. Studies with cells from myeloperoxidase-deficient patient. *J. Clin. Invest.*, **73**, 366-373.

Stevens, P. Winston, D. J. and vanDyke, K. (1978). *In vitro* evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states. *Infect. Immun.*, **22**, 41-59.

Trusch, M. A., Wilsson, M. E. and vanDyke, K. (1978). The generation of chemiluminescence by phagocytic cells. In *Methods in Enzymology*, Vol. LVII: p. 462.

Ward, P. A., Till, G. O., Kunkel, R. and Beauchamp, C. (1983). Evidence for the role of hydroxyl radical in complement and neutrophil-dependent tissue injury. *J. Clin. Invest.*, **72**, 789-801.

Williams, A. J. and Cole, P. J. (1981). The onset of polymorphonuclear leukocyte membrane stimulated metabolic activity. *Immunology*, **43**, 733-739.

Wymann, M. P., Tscharner, V., Deranleau, D. A. and Baggiolini, M. (1987). Chemiluminescence detection of H_2O_2 produced by human neutrophils during the respiratory burst. *Anal. Biochem.*, **165**, 371-378.