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SPIN TRAPPING OF SUPEROXIDE FROM GLASS ADHERENT POLYMORPHONUCLEAR LEUKOCYTES INDUCED BY N-FORMYLMETHIONYL-LEUCYL-PHENYLALANINE§

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Dahinden *et al.* reported that N-formylmethionyl-leucyl-phenylalanine (fMLP)-induced superoxide release from polymorphonuclear leukocytes (PMNs) lasted more than 60 min when the cells were allowed to attach to a petri dish before induction. In contrast, it lasted only for 2.5 min when cells were in suspension (*J. Clin. Invest.* 72: 113-121, 1983). In spite of this report, the effect of cell adhesion has been ignored in most spin trapping studies of superoxide release from PMNs. This study shows that most PMNs in a quartz flat electron paramagnetic resonance (EPR) cuvette which was placed horizontally adhered to the wall within 3 min. In contrast, if the cuvette was placed vertically, only 20-30% of the cells became adherent in 30 min. We performed spin trapping studies using 5,5-dimethylpyrroline-N-oxide (DMPO) as a spin trap, and monitored the effect of cell adhesion on superoxide generation. When spin trapping was conducted on PMNs in suspension, the EPR signal of superoxide adduct (DMPO-OOH) was undetectable after stimulation with fMLP. However, PMNs which were allowed to adhere to the cuvette after stimulation generated superoxide for hours. Moreover, when PMNs were allowed to adhere prior to the stimulation, the magnitude of superoxide release was augmented three-to fourfold. Unlike fMLP, phorbol myristate acetate (PMA), which has been most commonly used in spin trapping studies, induced superoxide release which was not influenced by cell adhesion. We emphasize the importance of specifying the cell-adhesion-state in spin trapping studies.

KEY WORDS: Spin trapping, PMN, fMLP, Cell Adhesion

INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are primarily important elements in host defense mechanisms against invading microorganisms. They are heavily armed with oxygen dependent and independent defense mechanisms, and the excessive or inappropriate liberation of oxygen radicals may cause tissue damage to the host.[1] Thus, regulation mechanisms which specify the targets of attack are critically important not only in terms of microbicidal efficiency, but also for prevention of adverse effects to the host.

Recent studies indicated that contact or adhesion of PMNs to particles such as

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bacteria, zymosan, crystals, various kinds of cells, and artificial or biological surfaces play key roles in the switching on and off the respiratory burst.[2,3] Some soluble stimuli like tumor necrosis factor-α (TNFα), TNFβ, colony stimulating factor-G (CSF-G) and CSF-GM only evoke superoxide release after cell adhesion.[3,4] Others like N-formylmethionyl-leucyl-phenylalanine (fMLP) or platelet activating factor (PAF) directly induce transient superoxide generation, but much more extensive generation occurs after cell adhesion.[3,5] Only non-physiological stimuli like phorbol myristate acetate (PMA) seem to provoke continuous superoxide release without cell adhesion.[3]

Spin trapping is a powerful tool in investigation of the respiratory burst of PMNs, because of its specificity for free radicals.[6] However, in most spin trapping studies, little attention has been paid to the state of cell adhesion. Consistent results have been obtained when PMA and opsonized zymosan have been employed as stimulants but not when other stimuli were used. The fact that the respiratory burst induced by PMA or opsonized zymosan is not influenced by the cell adhesion could explain this contradiction.

In the present spin trapping study we used a quartz flat cuvette and placed it in a horizontal orientation in an electron paramagnetic resonance (EPR) spectrometer. Under these conditions, the PMNs easily became adherent and it was found that the response of PMNs to fMLP stimulation was very dependent on the sequence of stimulation and adherence. We especially want to emphasize the importance of specifying the state of cell adhesion in the spin rapping studies on PMNs.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO). Dimethylpyrroline-N-oxide (DMPO) diluted to 1 M with Hanks' balanced salt solution (HBSS, pH 7.4), was washed with an equal volume of benzene twice, and the aqueous layer was filtered through a column packed with activated charcoal (15 mm i.d., 20 mm long) to remove impurities. FMLP and PMA were dissolved in dimethylsulfoxide (DMSO) at 1.0×10^{-2} M and 1.6×10^{-4} M, respectively, and were stored at $-70°C$ until used.

PMN Preparation

Heparinized venous blood from healthy volunteers was layered on Ficoll-Hypaque solution with a density of 1.112 g/ml. The solution was centrifuged at $500 \times g$ for 30 min, and the granulocyte layer was removed and suspended in HBSS. Contaminating erythrocytes were hypotonically lysed. After washing with HBSS, the PMNs were suspended in fresh HBSS.[7] More than 95% of the PMNs were neutrophils and the cell viability was greater than 98% by trypan blue exclusion.

Adhesion of PMNs to EPR Quartz Cuvette

A PMN suspension (200 μl, 10^6/ml) was transferred to a quartz EPR Cuvette (Wilmad WG814), and was placed in either a vertical or a horizontal position for various lengths of time. In order to wash out non-adherent cells, the cuvette was

placed vertically, and then 400μl of HBSS was gently introduced from the top end, carefully avoiding introduction of air bubbles. The effluent obtained from the bottom end was collected and the cell concentration was counted. The count obtained when cells were not allowed time to adhere was assumed to represent 0% adherence. The percentage of adherent cells was determined by comparing the cell concentration in the effluent with the 0% adherence value. The PMNs in the cuvette were observed using an inverted microscope (Olympus).

Spin Trapping and EPR

Three experimental designs were used to test the effect of cell adhesion on superoxide formation. In Method 1, the PMNs were stimulated and then transferred to the EPR cuvette. In Method 2, the PMNs were allowed to adhere first and then were stimulated. In this method, an EPR cuvette filled with a PMN suspension was placed horizontally for 10 min, followed by gentle washing with 400 μl of HBSS. Subsequently an aliquot of HBSS containing DMPO (100 mM), diethylenetriaminepentaacetic acid (DETAPAC, 100 μM), and DMSO solutions of either fMLP (10^{-5} M) or PMA (1.6×10^{-7} M) was introduced into the cuvette. The concentrations of stimulants resulted in maximal superoxide generation. In Method 3, PMNs were kept in the suspended state by agitation with a micro stirrer after stimulation in a polystyrene vial. Aliquots of the suspension were transferred periodically to an EPR cuvette, and the spectrum was recorded. In all methods, the final concentrations of DMPO, DETAPAC, fMLP, PMA were the same. The concentration of DMSO (solvent for fMLP and PMA) was 14 mM, which does not affect the respiratory burst. DETAPAC was added to prevent catalytic actions of metal irons in the medium.

EPR spectra were recorded on a Bruker 300E EPR spectrometer, to which a horizontal cavity was attached. The recording conditions were; magnetic field: 334.5 ± 5 mT, modulation frequency: 100 Hz, modulation amplitude: 0.1 mT, microwave frequency: 9.68 GHz, microwave power: 20 mW.

RESULTS

Adhesion of PMNs

As shown in the Figure 1, 80% of the PMNs became adherent to wall of a horizontally placed cuvette within 3 min after transfer. On the other hand, only 20–30% of the PMNs in a vertical cuvette became adherent in 30 min. Microscopic observation revealed that non-stimulated cells in a horizontally placed cuvette were almost all adherent to the glass wall. After stimulation with fMLP or PMA, cells spread on the glass, transforming their shape from round to flat. The spreading was more prominent after PMA stimulation. Neither stimulated nor non-stimulated adherent cells were removed by gentle washing with HBSS.

Spin Trapping Study

When PMNs were stimulated with fMLP and kept in the suspended state by agitation (Method 3), a weak EPR signal of DMPO-OH was observed, but signals of DMPO-OOH were absent (Figure 2C). When PMNs were stimulated and trans-

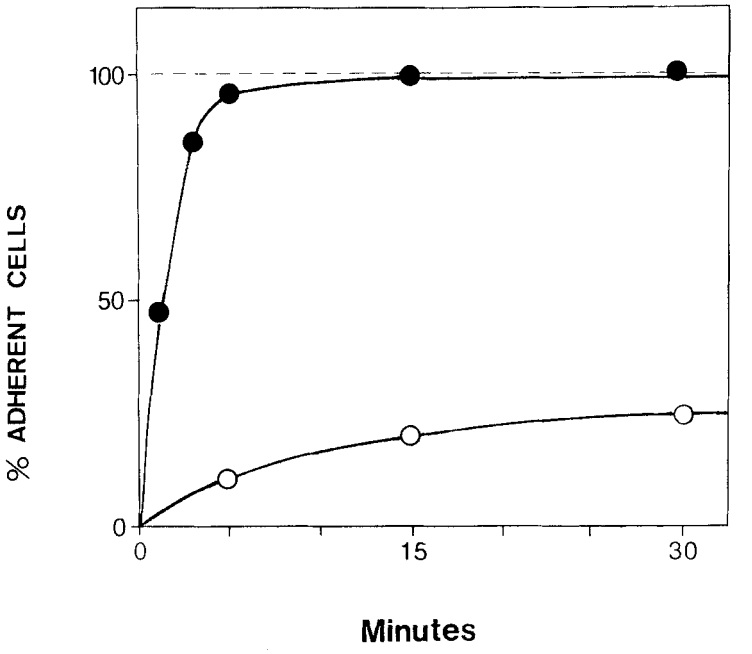

FIGURE 1 Time course of the percentage of adherent PMNs placed in EPR quartz cuvettes. The cuvette filled with PMN suspensions was placed in a vertical (○) or horizontal (●) position. After a given incubation time, the solution in the cuvette was drained and washed with 400 μl HBSS, and the cell concentration in the eluent was counted.

ferred to the cuvette (Method 1), DMPO-OOH signals were observed within 2 min (Figure 2B). When previously adherent PMNs were stimulated with fMLP (Method 2), the DMPO-OOH signal (Figure 2A) was three- to four-fold more intense than in PMNs which were allowed to adhere after stimulation (Figure 2B). The duration of the adhesion period (3–30 min) before stimulation with fMLP had no significant effect on DMPO-OOH signal (data not shown).

In the absence of fMLP, no signal of DMPO-OOH was observed, but weak signals of DMPO-OH were seen in Method 1 (Figure 2D), and this signal was inhibited by SOD (100 μ/ml), as reported by others.[8] Because glass contact alone can elicit superoxide release,[9] the small DMPO-OH signal may have arisen from DMPO-OOH which was below the limit of detection, and which was then degraded to the more stable DMPO-OH.[10] The significance of this adduct is hard to evaluate, because there are multiple pathways for its formation and its destruction is enhanced by superoxide.[11] Because the intent of this report is to focus on superoxide formation during the fMLP-induced respiratory burst, formation of secondary DMPO-OH will not be considered further.

The time course and the intensity of the DMPO-OOH adduct signal after fMLP stimulation were dependent on the state of cell adhesion and the sequence of adhesion and stimulation (Figure 3). In contrast, cell adhesion had little effect on superoxide generation after PMA stimulation (Figure 4). Although previous studies have shown that fMLP stimulation induced much weaker superoxide generation than was observed With PMA stimulation, in this study the maximal intensity obtained with

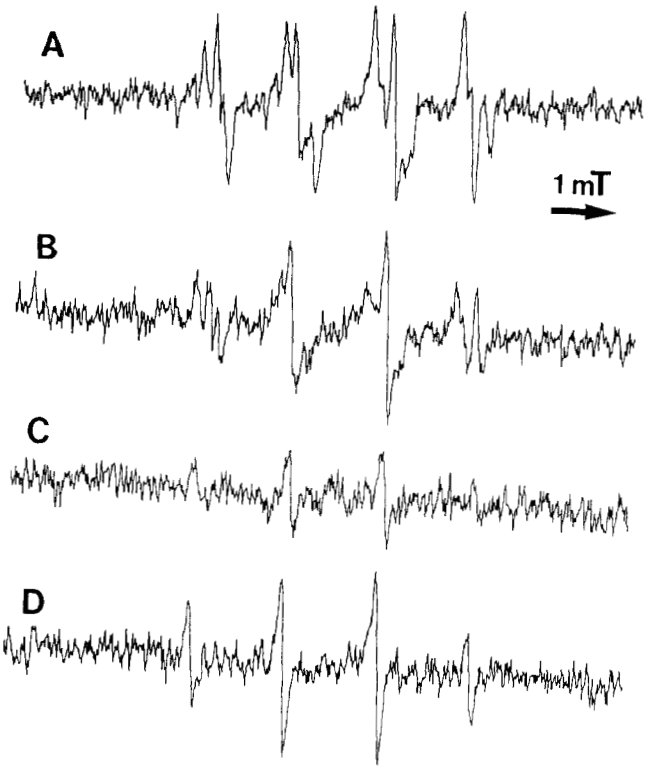

FIGURE 2 The EPR signal 4 min after stimulation with fMLP. A (Method 2): PMNs were adherent when stimulated, B (Method 1): PMNs were transferred to a cuvette after stimulation, C (Method 3): PMNs were kept in suspension for 3 min after stimulation, before transferring to the cuvette and scanning at 4 min, D: same as B except fMLP was omitted and the spectrum was recorded 60 min after transfer of cells into the cuvette.

fMLP reached 60% of that obtained with PMA (Figures 3 and 4). In similar experiments, we observed the DMPO-OOH signal for more than 2 hours after fMLP-stimulation, when Method 2 or Method 3 was used (data not shown).

DISCUSSION

As stated before, Dahinden *et al.* reported prolonged and pronounced generation of superoxide from fMLP-stimulated PMNs adhered on a petri dish.[3] A more recent study by Nathan confirmed this through a measurement of hydrogen peroxide generation by adherent PMNs on a laminin-coated plastic plate.[3] In that report, the amount of hydrogen peroxide generated after fMLP addition was comparable to that from PMNs stimulated with PMA,[3] which is very consistent with the present findings that adherent fMLP-stimulated cells generated demonstrated superoxide at nearly the same rate as PMA-stimulated cells. In the present experiments, the signal decrease started 30 min after stimulation in preadherent fMLP-stimulated PMNs as well as in PMA-stimulated PMNs. This may in part be

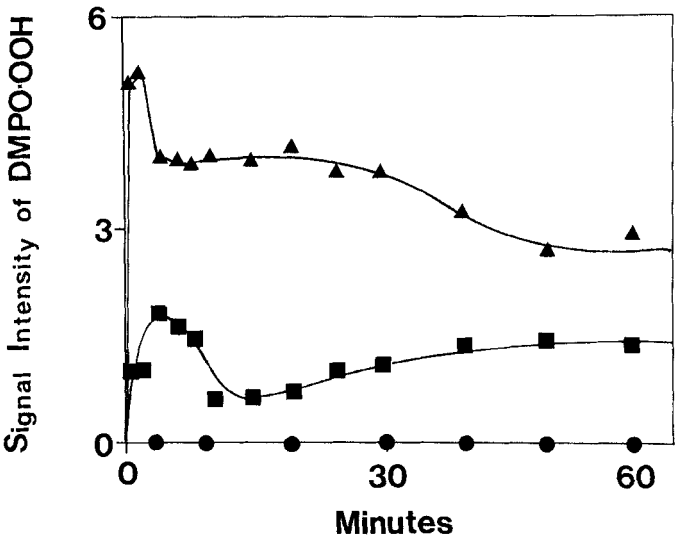

FIGURE 3 Time course of the signal intensity of DMPO-OOH obtained from fMLP-stimulated PMN. PMNs were kept in suspension (●) or were allowed to adhere to the glass cell after (■) or 10 min before (▲) stimulation. Signal intensities were expressed as arbitrary unit which was the same as in Fig. 4.

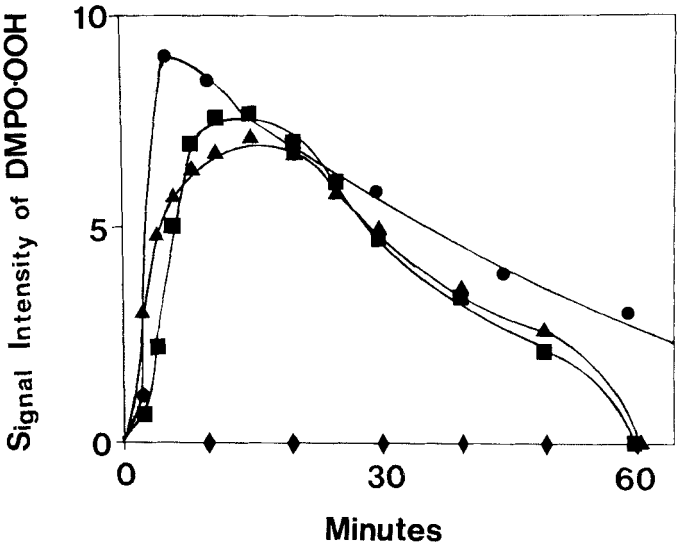

FIGURE 4 Conditions as in Figure 3, except the stimulant was PMA instead of fMLP. (♦) indicates results without PMA stimulation. Signal intensities were expressed as arbitrary unit which was the same as in Fig. 3.

attributed to a decrease in oxygen tension in the cuvette. Nevertheless, the comparison of DMPO-OOH signal intensities at the early stage of generation is valid.

Other investigators have also reported that adherence profoundly affects the ability of PMNs to respond to a variety of stimulating agents.[13-16] Among these

reports, there are results which contradict the present study, and indicate that fMLP stimulates superoxide generation by suspended cells to a greater extent than adherent cells.[14-16] For example, Hoffstein *et al.* compared superoxide generation by PMNs in petri dishes for 15 min in two different manners. They obtained higher superoxide generation from PMNs which were added to fMLP containing wells than from PMNs which were permitted to settle onto the surface before stimulation with fMLP. They concluded that adhesion attenuated superoxide generation by PMNs.[14] Rebut-Bonnton *et al.* also studied superoxide induced by fMLP from glass-adherent PMNs and suspended PMNs. They described rapid onset and short duration of the respiratory burst with both conditions, but the magnitude was greater in PMNs in suspension.[15] Laurent *et al.* compared superoxide release from PMNs suspended in plastic tubes and petri dish-adherent PMNs, and observed continuous superoxide generation for 2 hours in both experimental conditions. However, the magnitude of the response was larger in PMNs in the test tubes.[16]

A possible explanation for these contradictory findings could be differing cell densities. For example, the cell densities which we calculated from the literature were 1.6×10^6 cells/cm^2 for Hoffstein *et al.*, 6×10^5 cells/cm^2 for Rebut-Bonneton *et al.* and for Laurent *et al.*, whereas Nathan employed 5×10^4 cells/cm^2 and the current studies utilized 8.3×10^4 cells/cm^2. High cell population density is very likely to inhibit superoxide generation, because we found that cell-cell contact inhibits PMN-superoxide release.[17] This contact happens with adherent cells more readily than in cells with suspension. Furthermore, shorter observation periods could be another reason for underestimation of the amount of superoxide from adherent PMNs.[14,15]

In this study, we found that alteration of the sequence of adhesion and fMLP stimulation resulted in a great difference in the superoxide generation by PMNs. FMLP induced more superoxide generation from PMNs when cells were adherent to the glass before they were stimulated (Method 2), as compared with those stimulated followed by adhesion (Method 1). It is important to note that in both methods, PMNs were adherent and the fMLP concentration was the same. The results could be interpreted as an augmentation of the respiratory burst by cell adhesion, but this effect was attenuated by preexposure to fMLP.

FMLP has significant influence on cell adhesion. CD11b/CD18 (Mac-1, CR3) integrin has been identified as the major adhesion molecule in PMNs[18]. FMLP increases the number of CD11b/CD18 molecules[19] and also increases their activity.[20,21] Although glass adhesion and the resulting adhesion-induced respiratory burst are CD11b/CD18-independent,[9] there may be unidentified adhesion molecule(s),[9] and the enhancement of glass adhesion by fMLP[12,22] might suggest similar stimulatory effects of fMLP on the molecule(s). Adhesion in Method 1 and Method 2 might involve distinct but related processes.

PMA directly activates protein kinase C to initiate the respiratory burst, whereas fMLP interacts with membrane receptors and subsequent signal transduction triggers the respiratory burst. Because the extent of cell adhesion did not affect PMA-stimulated superoxide formation (Figure 4), the effects of cell adhesion observed with fMLP suggest that adhesion influences the signal transduction pathways. We hypothesize that cytoskeletal changes may be responsible for these effects. Southwick *et al.* reported that both fMLP and adhesion induced cytoskeletal changes, but the time courses were different[23]. FMLP induced a rapid rise of F-actin content which peaked after 30 s, and the level of F-actin subsequently declined to unstimulated levels within 20 min[23,24]. Unlike fMLP, adhesion induced actin

assembly more slowly. Maximum F-actin values were not observed until 10 min after adhesion, and the increased value remained constant over 60 min.[23] It has also been reported that the combination of adherence followed by fMLP resulted in an additional rise in F-actin content.[23] Applying these observations to the present results, both adhesion and fMLP might have acted in favor of actin polymerization in Method 2, whereas in Method 1, fMLP induced cytoskeletal change might have already progressed already in favor of depolymeryzation when cells adhered to glass.

Cytoskeletal reorganization has been implicated in the regulation of the respiratory burst in many respects. For example, alteration of fMLP-receptor affinity and decreased signal transduction from receptor-ligand complexes has been related to the binding of the receptors to the cytoskeleton.[25] Another example is that activation of NADPH oxidase involves assembly of membranous and cytosolic components, some of which are associated with the cytoskeleton.[26] Furthermore, the respiratory burst induced by adhesion to biological surfaces may also be influenced by cytoskeletal factors. Adhesion of PMNs to biological surfaces through CD11b/CD18 triggers the respiratory burst.[5,27] Hydrogen peroxide generation by TNF-stimulated PMNs was inhibited by monoclonal antibody to CD18 and also by the actin polymerization inhibitor cytochalasin B.[3,4,27]

Considering all described above, it seems probable that the different magnitude in superoxide release in Method 1 and Method 2 may be due to distinct cytoskeletal reorganizations which have taken place before both fMLP-stimulation and glass-adhesion were acquired, and the cytosleletal change in Method 2 allowed more efficient signal transduction to the respiratory burst. Clearly, further studies are necessary to elucidate the precise mechanisms involved.

Finally, we have reviewed published reports of PMN spin trapping studies in terms of the probable extent of cell adhesion. Flat quartz EPR cells have been employed in most spin trapping studies of PMN. When there is no special reference on the positioning,[8,10,11,28-39] it is assumed that the cuvette has been placed in its normal vertical orientation. According to our results, only 20–30% of cells become adherent within 30 min when a cuvette is kept vertical (Figure 1), and non-adherent cells did not produce DMPO-OOH after addition of fMLP (Figure 3). On the other hand, placing a cuvette in a horizontal direction brought about massive adhesion to the wall in a minute. If a special attention is not paid to the cuvette positioning throughout the experiment, varying amounts of PMNs may become adherent. Some investigators have used round Teflon tubes instead of flat quartz cells to take advantage of the oxygen permeability of Teflon,[11,40-42] and they also employed horizontal orientation of the cavity.[40-42] Although Teflon is less a stimulatory material than glass, stimulated PMNs should adhere to a Teflon surface as well.[9] In one EPR spin trapping study, macrophages were allowed to adhere to an EPR cuvette before stimulation.[43] In this study DMPO-OOH and DMPO-OH from Bacillus-Calmette-Guerin-activated macrophages after PMA stimulation were detected. Otherwise we could not find an article in which the state of adhesion is specified. The results of the present study emphasize the importance of adhesion in spin trapping studies of PMN.

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References

1. Anderson B.O., Brown, J.M. and A.H. Harken (1991) Mechanisms of neutrophil-mediated tissue injury. *Journal of Surgical Research*, **51**, 170–179, 1991.
2. J. Fehr, R. Moser, D. Leppert and P. Groscurth (1985) Anti-adhesive properties of biological surfaces are protective against stimulated granulocytes. *Journal of Clinical Investigation*, **76**, 535–542.
3. G.F. Nathan (1987) Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *Journal of Clinical Investigation*, **80**, 1550–1560.
4. G.F. Nathan (1989) Respiratory burst in adherent human neutrophils: Triggering by colony-stimulation factors CSF-GM and CSF-G. *Blood*, **73**, 301–306.
5. S.B. Shappel, C. Toman, D.C. Anderson, A.A. Taylor, M.L. Entman and C.W. Smith (1990) Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *Journal of Immunology*, **144**, 2702–2711.
6. B.E. Britigan, M.S. Cohen and G.M. Rosen (1987) Detection of the production of oxygen-centered free radicals by human neutrophils using spin trapping techniques: a critical perspective. *Journal of Leukocyte Biology*, **41**, 349–362.
7. A. Ferrante and Y.H. Thong (1982) Separation of mononuclear and polymorphonuclear leukocytes from human blood by one-step Hypaque-Ficoll method is dependent on blood column height. *Journal of Immunological Methods*, **48**, 81–85.
8. M.R. Green, H.A.O. Hill, M.J. Okolow-Zubkowska and A.W. Segal (1979) The production of hydroxyl and superoxide radicals by stimulated human neutrophils-Measarements by EPR spectroscopy. FEBS Letters, **100**, 23–26.
9. Y. Aida and M.J. Pabst (1991) Neutrophil responses to lipopolysaccharide. Effect of adherence on triggering and priming of the respiratory burst. *Journal of Immunology*, **146**, 1271–1276.
10. B.E. Britigan, G.M. Rosen, Y. Chai and M.S. Cohen (1986) Do human neutrophils make hydroxyl radical? *Journal of Biological Chemistry*, **261**, 4426–4431.
11. A. Samuni, C.D.V. Black, G.M. Krishna, H.L. Malech, E.F. Bernstein and A. Russo (1988) A Hydroxyl radical production by stimulated neutrophils reappraised. *Journal of Biological Chemistry*, **263**, 13797–13801.
12. C.A. Dahinden, J. Fehr and T.E. Hugle (1983) Role of cell surface contact in the kinetics of superoxide production by granulocytes. *Journal of Clinical Investigation*, **72**, 113–121.
13. M. Neuman and E. Kownatzki (1989) The effect of adherence on the generation of reactive oxygen species by human neutrophilic granulocytes. *Agents and Actions* **26**, 183–185.
14. S.T. Hoffstein, D.E. Gennaro and R.M. Manzi (1985) Surface contact inhibits neutrophil superoxide generation induced by soluble stimuli. *Laboratory Investigation*, **52**, 515–522.
15. C. Rebut-Bonneton, S. Bailly and C. Pasquier (1988) Superoxide anion production in glass-adherent polymorphonuclear leukocytes and its relationship to calcium movement. *Journal of Leukocyte Biology*, **44**, 402–410.
16. F. Laurent, A.M. Benoliel, C. Capo and P. Bongrand (1991) Oxidative metabolism of polymorphonuclear leukocytes: modulation by adhesive stimuli. *Journal of Leukocyte Biology*, **49**, 217–226.
17. T. Tanigawa, Y. Kotake and L.A. Reinke (1993), manuscript in preparation.
18. S. Albelda and C. Buck (1990) Integrins and other cell adhesion molecules. *FASEB Journal*, **4**, 2868–2880.
19. R.F. Todd III, M.A. Arnaut, R.E. Rosen, C.A. Crowly, W.A. Peter and B.M. Babior (1984) Subcellular localization of the large subunit of Mol (Molα; formerly gp110), a surface glycoprotein associated with neutrophil adhesion. *Journal of Clinical Investigation*, **74**, 1280–1290.
20. M.R. Philips, J.P. Buyon, R. Winchester, G. Weissmann and S.B. Abramson (1988) Up-regulation of the iC3b receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. *Journal of Clinical Investigation*, **82**, 495–501.
21. B. Schleiffenbaum, R. Moser, M. Patarroyo and J. Fehr (1989) The cell surface glycoprotein Mac-1 (CD11b/CD18) mediates neutrophil adhesion and modulates degranulation independently of its quantitative cell surface expression. *Journal of Immunology*, **142**, 3537–3545.
22. J. Fehr and C. Dahinden (1979) Modulating influence of chemotactic factor-induced cell adhesiveness on granulocyte function. *Journal of Clinical Investigation*, **64**, 8–16.

23. F.S. Southwick, G.A. Dabiri, M. Paschetto, S.H. Zigmond (1989) Polymorphonuclear leukocyte adherence induces actin polymerization by transduction pathway which differs from that used by chemoattractants. *Journal of Cell Biology*, **109**, 1561–1569.
24. T.H. Howard and C.O. Oresajo (1985) The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils. *Journal of Cell Biology*, **101**, 1078–1085.
25. A.J. Jesaitis, J.O. Tolley and R.A. Allen (1986) Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. *Journal of Biological Chemistry*, **261**, 13662–13669.
26. R.C. Woodman, J.M. Ruedi, A.J. Jesaitis, N. Okamura, M.T. Quinn, R.M. Smith T. Curnutte and B.M. Babior (1991) Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *Journal of Clinical Investigation*, **87**, 1345–1351.
27. C. Nathan, S. Srimal, C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit and S.D. Wright (1989) Cytokine-induced respiratory burst of human neutrophils: Dependence on extracellular matrix proteins and CD11/CD18 integrins. *Journal of Cell Biology*, **109**, 1341–1349.
28. R. Docampo, A.M. Casellas, E.D. Madeira, R.L. Cardoni, S.N.J. Moreno and R.P. Mason (1983) Oxygen-derived radicals from Trypanosoma cruzi-stimulated human neutrophils. *FEBS Letters*, **155**, 25–30.
29. B.E. Britigan, G.M. Rosen, B.Y. Thompson, Y. Chai and M.S. Cohen (1986) Stimulated human neutrophils limit iron-catalyzed hydroxyl radical formation as detected by spin-trapping techniques: effect of temperature and cellular metabolism. *Journal of Biological Chemistry*, **261**, 17026–17032.
30. G.M. Rosen, B.E. Britigan, M.S. Cohen, S.P. Ellington and M.J. Barber (1988) Detection of phagocyte-derived free radicals with spin trapping techniques: effect of temperature and cellular metabolism. *Biochimica Biophysics Acta*, **969**, 236–241.
31. S. Pou, G.M. Rosen, B.E. Britigan and M.S. Cohen (1989). Intracellular spin-trapping of oxygen-centered radicals generated by human neutrophils. *Biochimica Biophysica Acta*, **991**, 459–464.
32. S. Pou, M.S. Cohen, B.E. Britigan and G.M. Rosen (1989) Spin-trapping and human neutrophils. Limits of detection of hydroxyl radical. *Journal of Biological Chemistry*, **264**, 12299–12302.
33. B.E. Britigan and D.R. Hamill (1989) The interaction of 5,5-dimethyl-1-pyrroline-N-oxide with human myeloperoxidase and its potential impact on spin trapping of neutrophil-derived free radicals. *Archives of Biochemistry and Biophysics*, **175**, 72–81.
34. B.E. Britigan, D.J. Hassett, G.M. Rosen, D.R. Hamill and M.S. Cohen (1989) Neutrophil degranulation inhibits potential hydroxyl-radical formation. Relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques. *Biochemical Journal*, **264**, 447–455.
36. B.E. Britigan and D.R. Hamill (1990) Effect of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) on human neutrophil function: Novel inhibition of neutrophil stimulus-response coupling? *Free Radical Biology and Medicine*, **8**, 459–470.
37. M.S. Cohen, B.E. Britigan, Y.S. Chai, S. Pou, T.L. Roeder and G.M. Rosen (1991) Phagocyte-derived free radicals stimulated by ingestion of iron-rich Staphylococcus aureus: A spin-trapping study. *Journal of Infectious Diseases*, **163**, 819–824.
38. C.L. Ramos, S. Pou, B.E. Britigan, M.S. Cohen and G.M. Rosen (1992) Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. *Journal of Biological Chemistry*, **267**, 8307–8312.
39. I. Ueno, M. Kohno, K. Mitsuta, Y. Mizuta and S. Kanegasaki S (1989) Reevaluation of the spin-trapped adduct formed from 5,5-dimethyl-1-pyrroline-1-oxide during the respiratory burst in neutrophils. *Journal of Biochemistry*, **105**, 905–910.
40. A. Samuni, A. Samuni and H.M. Swartz (1989) The cellular-induced decay of DMPO spin adducts of $\cdot OH$ and $\cdot O_2^-$. *Free Radical Biology and Medicine*, **6**, 179–183.
41. A. Samuni, M. Krishna, P. Reisz, E. Finkelstein and A. Russo (1989) Superoxide reaction with nitroxide spin-adducts. *Free Radical Biology and Medicine*, **6**, 141–148.
42. C.D.V. Black, J.A. Cook, A. Russo and A. Samuni (1991) Superoxide production by stimulated neutrophils: temperature effect. *Free Radical Research Communications*, **12–13**, 27–37.
43. D.A. Hume, S. Gordon, P. J. Thornalley and J.V. Bannister (1983) The production of oxygen-centered radicals by Bacillus-Calmette-Guerin-activated macrophages. *Biochimica Biophysica Acta*, **763**, 245–250.

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