British Journal of Haematology, 1995, 89, 271–276

Correlations between the intensity of fMLP-dependent respiratory burst and cellular fatty acid composition in human neutrophils

P. BELLAVITE, P. GUARINI,* D. BIASI,* A. CARLETTO,* M. T. TREVISAN, P. CARAMASCHI,* L. M. BAMBARA* AND R. CORROCHER* *Institute of Clinical Chemistry and Microscopy and *Medical Pathology, University of Verona, Ospedale Policlinico, Verona, Italy*

Received 6 July 1994; accepted for publication 4 October 1994

Summary. The peripheral blood neutrophils were isolated from a group of normal subjects and their fatty acid composition determined by capillary gas-chromatography. The superoxide (O_2^-) release by the same cell preparations in response to formyl-methionyl-leucyl-phenylalanine was also determined following cytochrome c reduction in a microplate assay. A strong negative correlation was found between C18:2 (linoleic acid) ($r = -0{\cdot}703$, $P = 0{\cdot}001$) and C:16:0 (palmitic acid) ($r = -0{\cdot}569$, $P = 0{\cdot}009$) and fMLP-stimulated O_2^- release, whereas C20:4 (arachidonic acid) correlated positively ($r = 0{\cdot}448$, $P = 0{\cdot}048$). Other fatty acids, namely C12:0, C14:0, C16:1, C18:1, C18:3, C18:4, C20:0, C20:1, C20:2, C20:5, C22:0, C22:1, C22:6, C24:0 and C26:0, were not correlated with O_2^-. No correlations were found between fatty acid composition and O_2^- release from resting cells and from cells stimulated by phorbol-myristate acetate. These results suggest that the fatty acid composition of blood neutrophils may be a critical factor determining the capability of releasing free radicals in response to formylpeptides. Moreover, since a concomitant increase of arachidonic acid and decrease of its precursor linoleic acid has been found in high-responsive neutrophils, the rate of the enzymes of the arachidonic acid biosynthetic pathway (elongases and desaturases) appear to play an important role.

Keywords: fatty acids, neutrophil priming, superoxide release, signal transduction, phospholipids.

Neutrophils, stimulated by suitable doses of agonists and membrane perturbing agents, are affected by the activation of the enzymatic system NADPH oxidase, which generates excited oxygen species, including superoxide anion (O_2^-) and its derivatives hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$). These oxygen species participate in the bactericidal mechanisms of neutrophils, but also in the possible tissue damage caused by the disregulation of neutrophil activation in a number of pathologies (Sandborg & Smolen, 1988; Bellavite, 1988; Weiss, 1989; Segal & Abo, 1993). Because the duration and the magnitude of the metabolic response are important factors in determining the final balance – positive or unfavourable for the host – of neutrophil activation, these phenomena are subject to a fine and complex regulation at the level of receptors, transduction mechanisms, and NADPH oxidase itself.

Correspondence: Professor R. Corrocher, Istituto di Patologia Medica, Ospedale Policlinico, 37134 Verona, Italy.

In the course of studies on neutrophil activation and priming during inflammation (Bellavite *et al*, 1992, 1993; Biasi *et al*, 1993a, b) we noted that the extent of metabolic response to agonist challenge, in terms of superoxide formation, not only varied according to the functional state of the cells (i.e. if they were isolated from blood or from inflammatory exudates), but was quite heterogenous in a population of normal subjects, even considering the blood cells only. Some of the variability could be due to technical reasons such as the procedures of cell purification, but as a broad distribution of superoxide levels has also been found previously in whole blood measurements (Bellavite *et al*, 1983), it is more conceivable that the heterogeneity is due to the complex and multiple-step nature of NADPH oxidase activation (McPhail *et al*, 1984; Maridonneau-Parini *et al*, 1986; Bellavite, 1988; Bellavite *et al*, 1990). According to the current knowledge of neutrophil regulation, a conceivable and relevant mechanism explaining interindividual differences of the oxidative metabolism could be the different lipid composition of membranes, due to diet, lifestyle, age, sex,

genetic background, or other unknown factors. In fact, several lines of evidence indicate that lipid composition of the cell membrane may have an important role in the regulation of the respiratory burst of neutrophils. Certain saturated fatty acids (myristate in particular) and *cis*-unsaturated fatty acids (arachidonate in particular) elicit production of O_2^- in intact cells (Kakinuma, 1974; Curnutte *et al*, 1984). A direct role of lipids in the regulation of activation or activity of the free-radicals-generating oxidase has been suggested by the stimulating effect on the solubilized and isolated enzyme exhibited by phosphatidylethanolamine (Gabig & Babior, 1979), by phosphatidylserine (Tamura *et al*, 1988) and by phosphatidic acid (Bellavite *et al*, 1988). Moreover, the NADPH oxidase in cell-free systems is activated by arachidonic acid and other unsaturated fatty acids (Bromberg & Pick, 1984). Finally, it has been shown recently that arachidonic acid acts also directly on the assembled NADPH oxidase in the membranes and in the endosomes and increases its catalytic activity by increasing the affinity for NADPH (Rubinek & Levy, 1993).

These data and observations prompted us to investigate the relationship between lipid composition and oxidative metabolic responses in a simple and straightforward way, i.e. by measuring, in a group of normal human subjects, the amount of neutrophil O_2^- production and the amount of the most representative species of fatty acids contained in the same cells. To the best of our knowledge, no similar data are available in the literature.

Neutrophils were isolated from peripheral blood, then divided in two aliquots, one of which was used for the determination of fatty acids, the other was used for superoxide production in a microplate assay. Superoxide production was measured on resting cells, on cells stimulated by an agonist that acts through specific membrane receptors, namely n-formyl-methyonyl-leucyl-phenylalanine (fMLP) and on cells stimulated by phorbol-myristate acetate (PMA), an agent which is known to perform its effects through direct stimulation of protein kinase C. Both positive and negative correlations between the two considered parameters emerged from our study.

METHODS

Reagents. FMLP, PMA and reference fatty acids were purchased from Sigma Chemical Company, St Louis, Mo.; cytochrome c from Boehringer, Mannheim, Germany; purified serum bovine albumin and human albumin from Behring Institut, Marburg, Germany; Percoll was from Pharmacia, Uppsala, Sweden. Sterile 96-well microtitre plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. The microplates were pre-coated with fetal bovine serum (Flow Laboratories) in order to abolish nonspecific cell activation as described (Bellavite *et al*, 1992). Hanks balanced salt solution (without calcium and magnesium) (HBSS) and Dulbecco's phosphate-buffered saline (PBS) were from Gibco Ltd, Paisley, Scotland; the composition of HBSS was 0·4 g/l KCl, 0·06 g/l KH_2PO_4, 8 g/l NaCl, 0·09 g/l Na_2HPO_4, 1 g/l D-glucose (pH 7·4); the composition of PBS was 0·2 g/l KCl, 0·2 g/l KH_2PO_4, 0·047 g/l $MgCl_2$, 8 g/l NaCl, 1·15 g/l Na_2HPO_4 (pH 7·4). Other materials and reagents were of the highest purity available.

Cell preparation. Neutrophils were obtained from blood of normal human volunteers by centrifugation over discontinuous Percoll gradients (Metcalf *et al*, 1986). Ethylene diamine tetraacetate-anticoagulated blood samples were centrifuged over Percoll gradients for 20 min at 1800 rpm with a Sorvall T6000B centrifuge. Neutrophils were recovered as a broad band at the 73%–62% interface, were diluted with 1 volume of PBS, and centrifuged for 10 min at 1200 rpm. The pellet was usually slightly contaminated by erythrocytes, that were lysed by a brief hypothonic shock: cells were suspended in 5 ml of 0·2% NaCl for 20 s, then the isotonicity was restored by addition of 5 ml of 1·6% NaCl and right pH was restored by addition of 2 ml of PBS. After hypotonic lysis of contaminating erythrocytes and two washings with PBS, the cells (>95% neutrophils, >99% viable as judged by trypan blue exclusion test) were finally suspended in HBSS, containing 0·2% human serum albumin (H-A), and kept at room temperature until use. A few minutes before use, 100× concentrated solutions of $CaCl_2$ and $MgSO_4$ were added to the cell suspensions at the final concentration of 0·5 mM and 1 mM respectively.

Superoxide measurement. A microplate assay of O_2^- production was performed according to previously reported procedures (Bellavite *et al*, 1992, 1993), with the following modifications. The assay medium was H-A supplemented with 0·5 mM $CaCl_2$ and 1 mM $MgSO_4$ (H-ACM). The microplate wells were supplemented with 25 μl of 0·6 mM cytochrome c and with either 25 μl of the stimulant dissolved in H-ACM (fMLP or PMA at the final concentration of 5×10^{-7} M and 10 ng/ml respectively) or with 25 μl of H-ACM (resting assays). Assays were currently done in triplicate for each experimental condition. The plate was brought to 37°C, and 50 μl of the neutrophil suspension (2×10^5 cells), pre-warmed at 37°C, were added to each well. The plate was then incubated for 10 min in a humidified thermostat at 37°C. When required, the plate was rapidly transferred into a microplate reader (Reader 400, SLT Labs Instruments) and the reduction of cytochrome c was measured at 550 nm using 540 nm as reference wavelength, and using 0·037 optical density units as standard for 1 nmol of reduced cytochrome c (Bellavite *et al*, 1992).

Fatty acid determination. Analysis of fatty acids was performed on aliquots of neutrophil preparations containing 10^7 cells, by extraction of total lipids with 4·5 ml of isopropanol/chloroform (11/7, v/v) added with 0·45 mM 2,6-di-ter-*p*-cresol (BHT) as antioxidant. A gas chromatographic (Hewlett Packard 5890 chromatograph; Hewlett Packard, Palo Alto, Calif., U.S.A.) method was employed as previously described (Girelli *et al*, 1992), based on fatty acid direct transesterification technique (Lepage & Roy, 1986). Analysis were performed in duplicate on each sample. Peak identification and quantification were made with reference fatty acids commercially available. As internal standard, heptadecanoic acid (C17:0) was used. The heights of the peaks were measured and the quantification was performed

with the aid of a PC Vectra (QS/16S equipped with the software HP-3365 ChemStation Hewlett Packard) working in Microsoft Windows 3.0. Fatty acid composition data were expressed as mg/100 mg fatty acid methyl esters. The mean percentage recovery was 90·6 ± 2·7 (range 85–94%).

Data analysis. Statistical analysis was performed on a PC Apple Macintosh SE/30 equipped with the software Systat 5.0, working in Windows 3.0 (Microsoft). Values are expressed as mean ± standard deviation and the correlation between different parameters was studied using the Pearson correlation coefficient.

RESULTS

The mean fatty acid compositions of neutrophils from the group of 20 normal volunteers used in this study are given in Table I. It can be seen that 44% of fatty acids were saturated, the most represented being palmitic and stearic; 30% were monounsaturated, represented almost exclusively by oleic acid; a quarter of fatty acids were polyunsaturated and, among these, 9% was represented by linoleic acid and 13% by arachidonic acid.

The O_2^- release by the neutrophils (nmol/10 min/10^6 cells, $n = 20$) was as follows: resting cells, 0·92 ± 0·82 (range 0·0–3·0); fMLP-activated cells, 11·04 ± 3·55 (range 6·0–17·0); PMA-activated cells 28·33 ± 9·4 (range 19·5–49·6). The fatty acid content of blood neutrophils was then plotted against the production of superoxide. The oxidative metabolism of resting (unstimulated) cells was so low that no possible correlations could be calculated with the fatty acids content. Therefore the only consistent data were obtained on fMLP- and PMA-activated cells and the correlation drawn was between fatty acid content and the activation of the metabolism. Among the fatty acids species reported in Table I, the majority were not correlated with oxidative metabolism and nor was correlation found with the total content of unsaturated and saturated fatty acids (not shown). However, three fatty acid species showed a significant correlation with fMLP-activated respiratory burst

Table I. Fatty acid content of neutrophils from 20 normal subjects.

	Total fatty acids (w/w) (%) ±SD
Saturated	
Total	44·40 ± 2·35
C12:0	0·27 ± 0·15
C14:0	0·77 ± 0·23
C16:0 (palmitic)	21·35 ± 1·19
C18:0 (stearic)	18·84 ± 2·19
C20:0	0·38 ± 0·02
C22:0	1·28 ± 0·19
C24:0	1·92 ± 0·26
C26:0	0·37 ± 0·14
Monounsaturated	
Total	29·91 ± 2·74
C16:1w7	0·52 ± 0·24
C18:1w9 (oleic)	27·94 ± 2·19
C20:1w9	1·01 ± 0·23
C22:1w9	1·34 ± 0·51
Polyunsaturated	
Total	24·93 ± 1·93
C18:2w6 (linoleic)	9·32 ± 1·71
C18:4w3	1·22 ± 0·24
C20:2w6	0·98 ± 0·29
C20:4w6 (arachidonic)	12·81 ± 1·63
C20:5w3	0·60 ± 0·03
C22:6w6	1·26 ± 0·32

(Fig 1). These were palmitic acid and linoleic acid which showed a negative correlation, and arachidonic acid which showed a weak, but significant, positive correlation. In other words, the superoxide production was higher in cells having a low content of palmitic and linoleic acid and a high content of arachidonic acid.

It should be pointed out that the determinations of superoxide production and of fatty acids were done by two

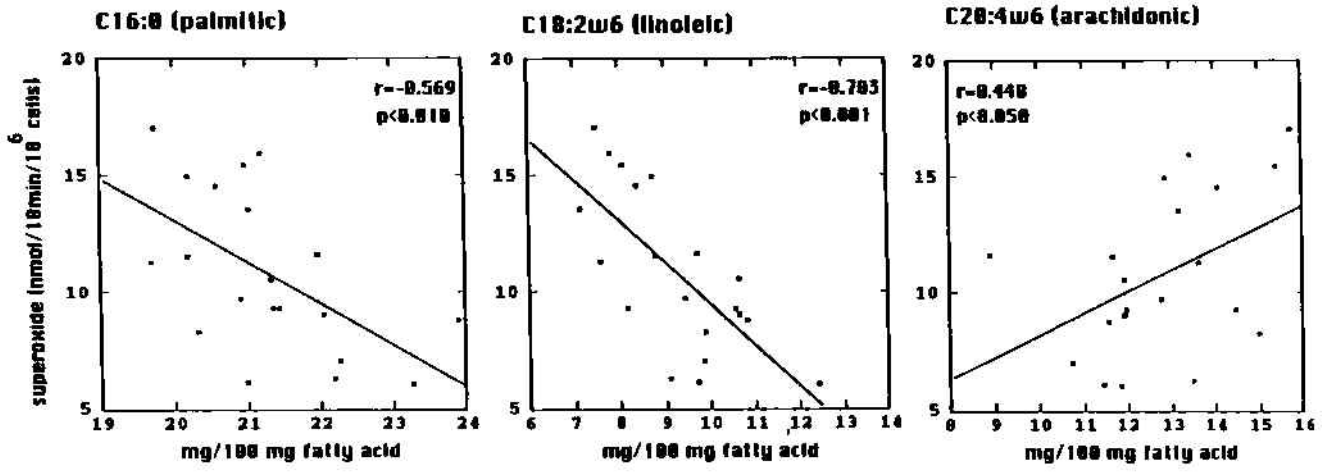

Fig 1. Linear regression plot of fatty acid content and fMLP-stimulated O_2^- release in human neutrophils.