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ORIGINAL ARTICLE

A. Choukèr · A. Martignoni · L. DaSilva · H. Diem
F. Christ · K. Peter · M. Thiel

Cryopreservation of reduced cytochrome C for determination of N-formyl-methionyl-leucyl-phenylalanine-stimulated superoxide anion production in human whole blood

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Abstract Various methods are available for measuring the production of reactive oxygen species by phagocytes, but they are limited in their use by the need for their immediate application, cell isolation and of cell-activation by unphysiological stimuli. In addition, after measurement of reactive oxygen metabolites using oxidizing agents, the reduced compounds formed have to be determined during or immediately after their formation. In the present study, an improved cytochrome C assay was investigated which allowed measurements of superoxide anions in whole blood samples following activation of phagocytes by physiological stimuli such as the bacterial tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). The fMLP-stimulated production of superoxide anion (O_2^-) showed a sigmoidal-shaped fMLP dose-response curve, and constant O_2^- production rates (nmol·l^{-1}×10^6 granulocytes) could be determined reliably up to a blood granulocyte concentration of $1\times10^4\cdot\mu l^{-1}$. To allow the determination of reduced cytochrome C later after its formation, the effect of long-term storage at –20°C on the stability of reduced cytochrome C was tested up to 16 weeks. The results obtained show that the determination of reduced cytochrome C in whole blood represents a simple and reliable method. Most importantly, O_2^--reduced cytochrome C can be frozen and stored without any alterations, at least up to 2 weeks. Thus the method seems to be superior to other methods of detection, especially when the experimental conditions do not allow immediate spectrophotometry (e.g. mountain medicine, space medicine). Under such conditions the present assay would allow reliable measurement of reduced cytochrome C, even after weeks of cryopreservation.

Keywords Polymorphonuclear leucocytes · Superoxide anions · Cytochrome C · Cryopreservation

A. Choukèr (✉) · A. Martignoni · L. DaSilva · F. Christ
K. Peter · M. Thiel
Clinic of Anaesthesiology,
Klinikum Großhadern,
Ludwig-Maximilians-University of Munich,
81366 Munich, Germany
E-mail: alexander.chouker@ana.med.uni-muenchen.de
Tel.: +49-89-70956422
Fax: +49-89-70958886

H. Diem
Institute of Clinical Chemistry,
Klinikum Großhadern,
Ludwig-Maximilians-University of Munich,
81366 Munich, Germany

Introduction

Polymorphonuclear leucocytes (PMNL) constitute the first line of defence against invading bacterial microorganisms. To this end, PMNL produce substantial quantitites of superoxide anions (O_2^-) and other bacteriocidal reactive oxygen species. On the other hand, the formation of highly toxic oxygen radicals can also cause endothelial cell damage and tissue injury by inflammatory processes. Among several methods used for the determination of superoxide anions (McNeil et al. 1989; Vasquez Vivar et al. 1997) the reduction of cytochrome C (cyC) was established by Babior (Markert et al. 1984). However, this assay usually requires time-consuming steps to isolate PMNL from whole blood, thereby altering the response of the cells. For example, the commonly used dextran solution for sedimentation of red blood cells has previously been demonstrated to cause spontaneous depolarization of the cell membrane (Seeds et al. 1985). Most importantly, isolation of leucocytes by gradient centrifugation may cause significant upregulation of cell-surface adhesion receptors (Fearon and Collins 1983), an effect which primes phagocytes for enhanced oxidative responses (Richter 1992). All these effects induced by cell isolation, lead to an altered cellular functional state which may not reflect the functional behaviour of the cells in vivo. To avoid isolation of PMNL, we measured the reduction of cyC in diluted whole blood samples following the activation of phagocytes by the receptor-dependent, physiological

stimulus N-formyl-methionyl-leucyl-phenylalanine (fMLP). The whole-blood assay was characterized with respect to the dose-response for fMLP and the dependency of the chemotactically induced O_2^--production on the number of PMNL. Moreover, we centrifuged reaction vials after the activation of PMNL by fMLP was completed and the supernatants were collected and frozen to examine the influence of storage for different time intervals on the determination of the O_2^--production by reduced cyC.

Methods

Volunteers

The volunteers participating in the study were men whose mean (SEM) age is given in the legends of the tables and figures. The study protocol was approved by the Ethics Committee of the Ludwig-Maximilians-University of Munich and all the in vitro experiments complied with the current German law. Written and informed consent was obtained from the volunteers prior to blood collection.

Whole-blood assay

A total of 1,000 µl of freshly drawn heparinized whole blood was divided among four plastic tubes all prefilled with pre-warmed (37°C) reaction mixture of 1,400 µl of Hank's buffered salt solution (HBSS), cyC (final concentration: 0.625 $mg \cdot ml^{-1}$, 50 $\mu mol \cdot l^{-1}$), cytochalasin B (final concentration: 2.5 $\mu g \cdot ml^{-1}$, 50 $\mu mol \cdot l^{-1}$). One tube had no further additives, while the other contained superoxide dismutase (SOD: final concentration 50 $U \cdot ml^{-1}$) or fMLP with the latter in the absence and presence of SOD. In total, each vial contained 1,667 µl. These reaction mixtures were incubated at 37°C for 15 min, and the cellular components were separated (5 min, 600 *g*). Thereafter, the supernatants were collected and transferred to a microtitre plate, and the absorbances were determined in triplicate on a multi-channel automated photometer (Dynatec MRX 7000, Dynatec Laboratories Inc., Alexandria, Va., USA). Plate readings were made at 550 nm fitted with a 630 nm interference filter. The path-length in the microplate containing 180 µl supernatant was 0.35 cm. From the difference between the absorbances determined in the samples stimulated with fMLP in the absence and in the presence of SOD, the amount of superoxide anion (O_2^-) generated in response to the chemotactic tripeptide was calculated, as previously described (Markert et al. 1984). Spontaneous production of O_2^- was determined from the differences in the absorbances of unstimulated samples with and without SOD. The results are expressed as nanomoles of O_2^- formed per 1×10^6 phagocytes and per 15 min. Cell counts were done by Coulter counter measurements (Coulter Multisizer, Coulter Electronics Ltd., Luton, UK). The HBSS was produced by the institution's own pharmacy and contained the following substances in 1,000 ml: NaCl (8.0 g), glucose-monohydrate (1.0 g), KCl (400 mg), $MgCl_2$ (74 mg), $MgSO_4$ (74 mg), $CaCl_2$ ((138 mg), KH_2PO_4 (60 mg), Na_2HPO_4 (150 mg), $NaHCO_3$ (300 mg). The chemicals used were of analytical grade and were purchased from Sigma Chemical (Deisenhofen, Germany).

Assay with isolated PMNL

Granulocytes were separated from whole blood of healthy volunteers by a modified method previously described by Boyum (1968). Heparinized whole blood [10 i.u.(international units) $heparin \cdot ml^{-1}$, Braun Melsungen, Melsungen, Germany) was mixed with an equal volume of dextran 60 (Macrodex 6%, Pharmacia Fine Chemicals, Uppsala, Sweden) in NaCl (0.9%). After erythrocyte sedimentation for 45 min the leucocyte-enriched supernatant was removed and centrifuged (600 *g*, 10 min) on a discontinuous Ficoll-Histopaque gradient (density: 1,077 $mg \cdot ml^{-1}$). The PMNL fraction pelleting at the bottom of the tube was harvested. The PMNL were washed twice with HBSS and contaminating red blood cells (RBC) were lysed by hypotonic shock in distilled water for 30 s. Normal osmolarity was restored by the addition of an equal volume of hypertonic NaCl solution (1.8%). Isolated PMNL were washed twice and resuspended in HBSS. The capacity of separated PMNL to produce O_2^- was determined under the same conditions of incubation as described for the whole-blood assay, using instead of whole blood a comparable number of isolated PMNL.

Dose-dependent effects of increasing fMLP concentrations on O_2^--formation in whole blood and isolated PMNL

The effects of increasing concentrations of fMLP (10^{-10} $mol \cdot l^{-1}$ to 10^{-5} $mol \cdot l^{-1}$) on the production of O_2^- from phagocytes in whole blood and from phagocytes previously isolated from the blood were evaluated under the same experimental conditions as described above.

β2-integrins

To estimate PMNL-activation due to cell separation procedures (dextran 60 sedimentation, Ficoll gradient centrifugation, hypotonic lysis of RBC) cell-suspensions of separated PMNL were incubated with fluorescein isothiocyante (FITC)-labelled monoclonal antibody IB4 which specifically binds to β2-integrins (CD18). Fluorescence intensities were analysed using a FACScan (Becton Dickinson, San Jose, Calif., USA). The expression of adhesion molecules was thereafter calculated as previously described (Thiel et al. 1996) and expressed as relative fluorescence units. The results were compared to the β2-integrin expression of untreated PMNL in diluted whole blood samples.

Correlation between PMNL blood concentration and fMLP-stimulated O_2^- production, proof of linearity

To verify a possible relationship between the number of phagocytes and the amount of O_2^- formation in whole blood, 33 different blood samples from healthy volunteers were analysed. The normally distributed PMNL concentrations (1,000–6,000 $PMNL \cdot \mu l^{-1}$) from the same subjects were correlated to the O_2^- formation of phagocytes in response to fMLP-stimulation. However, looking for a correlation between O_2^- formation and numbers of PMNL, especially when their counts are in the normal physiological range, might not be useful in proving assay reliability at higher PMNL concentrations. To overcome this problem, PMNL from the blood of the same volunteers were previously isolated and added to the whole-blood assay. Assuming that the PMNL added to the assay might have been activated due to separation procedures, the results can only indicate a relationship between the rate of O_2^- production and higher PMNL-counts up to 22,000 $PMNL \cdot \mu l^{-1}$.

Effects of long-term cryopreservation on cyC reduced by O_2^- generated in whole blood

A total of 1,000 µl of heparinized whole blood was split into four portions and incubated as described above (fMLP 10^{-6} $mol \cdot l^{-1}$). After incubation at 37°C for 15 min, the reaction mixtures were centrifuged (5 min, 600 *g*). Thereafter, the cell free supernatants were withdrawn and divided into four aliquots (one for immediate determination, the remaining three aliquots for determination after cryopreservation at –20°C for 2, 8 or 16 weeks).

Interference of free haemoglobin with the photometric measurements

Because free haemoglobin might interfere with photometric measurements (wavelength of maximal absorbance of oxy-and deoxy-haemoglobin is near the wavelength used for the detection of reduced cyC), cell-free supernatants of whole-blood assays without prior addition of cyC were assessed for absorbance by photometry.

Cell-counts

For all experiments the concentrations of PMNL were determined from EDTA-anticoagulated whole-blood specimens.

To determine the effects of time on neutrophil counts in blood specimen, white blood cell counts were determined in blood samples at 0, 8, 16, 24, 48, 72 or 96 h after blood acquisition (Coulter STKS, Coulter Electronics Ltd.). In addition, blood smears were made immediately after blood acquisition from the same subjects.

Statistics

All data were tested for normal distribution by the Kolomogorov-Smirnov test and analysed by paired Student's *t*-tests (SPSS V.10, SPSS Inc., Chicago, Ill., USA). In the case of multiple comparisons, levels of significance were corrected according to Bonferroni. Bivariate correlation by Pearson product moment correlation was used to measure in young and healthy volunteers the strength of association between normally distributed PMNL counts and the rate of the fMLP-stimulated O_2^- production. Linear regression analysis was used to study the relationship between PMNL number and chemotactically stimulated O_2^- production when absolute PMNL counts were increased by the addition of isolated PMNL. To evaluate the precision of the photometric measurements, the *intra*-assay ($n=24$) and *inter*-assay ($n=7$) coefficients of variation (CV) were determined. The CV is given by the ratio of the standard deviation (SD) and the mean value. The *intra*-assay CV determines the precision of simultaneously taken measurements of the same sample distributed in the wells of one microtitre plate. The *inter*-assay CV describes the reproducibility of measurements of the same sample assayed in separate assays in different microtitre plates. For the determination of CV values, the cell-free supernatants obtained from one whole-blood experiment were used.

Results

Incubation of diluted whole-blood and isolated PMNL with increasing concentrations of fMLP

As shown in Fig. 1, in the whole-blood assay very low concentrations of fMLP (1×10^{-10} mol·l^{-1} – 1×10^{-8} mol·l^{-1}) did not cause any detectable increase of the O_2^- production. Upon stimulation of granulocytes with further increasing concentrations of fMLP (10^{-7} mol·l^{-1} – 10^{-5} mol·l^{-1}), maximal rates of O_2^- production were obtained.

Isolated PMNL already showed a higher unstimulated basal level of O_2^- production and a significant increase at the very low fMLP concentrations (1×10^{-9} mol·l^{-1} – 1×10^{-8} mol·l^{-1}). A comparision of the dose-response curves of whole-blood and isolated PMNL showed that they were both sigmoidal with the maximal stimulation at a fMLP concentration of 10^{-6} mol·l^{-1}. No further rise in the production of O_2^- occurred when fMLP concentrations were increased to 10^{-5} mol·l^{-1}.

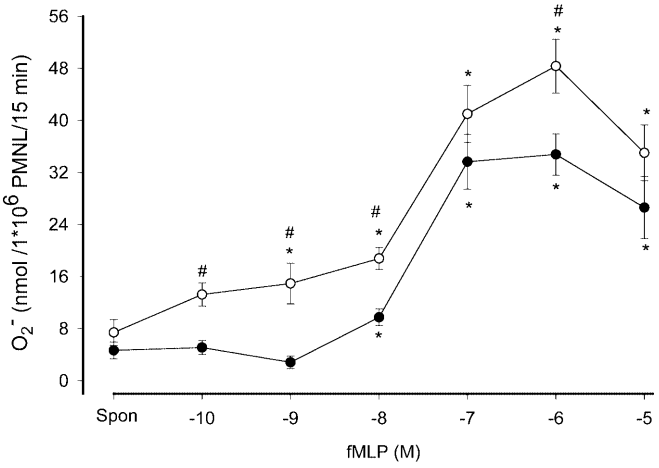

Fig. 1. Dose-response curves of the effect of increasing concentrations of N-formyl-methionyl-leucyl-phenylalanine (*fMLP*) on the superoxide anion (O_2^-) production in diluted whole blood (*filled circles*) and isolated polymorphonuclear leucocytes (*PMNL*, *open circles*). The whole blood samples and the isolated PMNL were incubated separately with a reaction mixture containing cytochrome C (0.625 mg·ml^{-1}, 50 μmol·l^{-1}), cytochalasin B (2.5 μg·ml^{-1}, 50 μmol·l^{-1}) and Hank's buffered salt solution. After 15 min of incubation at 37°C fMLP was added at increasing concentrations in the absence or presence of superoxide dismutase (SOD) (50 U·ml^{-1}). Absorbances were read at 550 nm and the SOD-inhibited O_2^--production was calculated as described in the Methods. The dose-response curves were sigmoid in shape in whole blood as well as for isolated PMNL, reaching maximal effects at concentrations of fMLP of 10^{-7} and 10^{-6} mol·l^{-1}. The fMLP-mediated production of O_2^- in isolated PMNL could be induced at lower concentrations of fMLP (10^{-9} mol·l^{-1}) compared to whole blood PMNL. Volunteers' mean (SEM) age 29.6 (1.1) years, Student's *t*-test (two sided), $*P<0.05$ compared to spontaneous activity (*Spon*), $\#P<0.05$ compared to whole blood; $n=9$

Effects of PMNL isolation on O_2^- production and expression of β2-integrins

As shown in Table 1, when the O_2^- production elicited by the maximal concentration of fMLP (10^{-6} mol·l^{-1}) was compared between the whole-blood specimen and

Table 1. Effects of polymorphonuclear leucocytes (*PMNL*)-isolation on superoxide anion (O_2^-) production and expression of β2-integrins. Previous isolation of PMNL from the blood of healthy volunteers led to PMNL activation as seen by the increased rate of O_2^- production [nmol·1^{-1}×10^6 PMNL, N-formyl-methionyl-leucyl-phenylalanine (*fMLP*) 10^{-6}] and enhanced expression of β2-integrins (CD18) on separated cells compared to the respective values of PMNL in whole blood [volunteers' mean (SEM) age = 31.45 (1.7) years, $n=5$). *rel fl* Relative fluorescence

		O_2^- Production (fMLP 10^{-6} mol·l^{-1}) (nmol·1×10^6 PMNL·15 min^{-1})	CD 18 (rel fl units)
Blood	Mean	35.87	31.36
PMNL	SEM	3.82	7.09
Isolated	Mean	52.11*	42.25*
PMNL	SEM	5.45	8.05

*t-test (one-sided) $P<0.05$ compared to blood PMNL

isolated PMNL, the maximal activity of isolated PMNL was up to 30% higher than that obtained for PMNL in whole blood. In addition, in resting cells, i.e. not subjected to fMLP-stimulation, the expression of $\beta 2$-integrins (CD18) on the PMNL surface was significantly higher compared with cells in whole blood.

Relationship between PMNL number per microlitre of whole blood and the rate of fMLP-stimulated O_2^- production

As shown in Fig. 2 and Table 2, when PMNL blood concentrations in the normal physiological range were tested for a relationship to the rate of O_2^- production (nmol·1^{-1}×10^{-6} PMNL·15 min^{-1}) no significant correlation could be observed ($r = 0.21$, $P = 0.22$, Pearson's correlation coefficient; Fig. 2A). However, the physiological range of granulocyte counts did not exceed 6,000 PMNL·μl^{-1}. To test a possible relationship between a higher number of PMNL (more than 6,000·μl^{-1}) in the whole-blood assay and the production of O_2^-, increased numbers of separated PMNL were added to the whole-blood assays. As a result, the calculated rates of O_2^- production remained constant at 30–40 nmol·1^{-1}×10^6 PMNL·15 min^{-1} over a vast range up to high granulocyte total cell counts of 1×10^4 PMNL·μl^{-1} in the blood (Fig. 2B). Linear regression analysis up to 1×10^4 PMNL·μl^{-1} yielded the following equation ($y = y0 + ax$): O_2^-(nanomoles) = 35,502 + (–3,323×10^{-4})PMNL·μl^{-1} blood, $r = -0.15013$). However, when the number of PMNL per microlitre whole blood was greatly increased from 1.1×10^4 to more than 2.2×10^4 the fMLP-stimulated O_2^- production rate decreased [O_2^-(nanomoles) = 43.23 + (–1.46×10^{-3}) PMNL·μl^{-1} blood, $r = -0.78$] (Table 2).

Long-term cryopreservation

We next studied the effects of long-term cryopreservation on the stability of reduced cyC sampled from the whole-blood assay. When cell free supernatants were frozen at –20°C and thawed after 2 (T1) weeks, the values measured were not significantly different from those determined immediately before freezing (T0) (Fig. 3). In contrast after 8 (T2) and even more after 16 weeks (T3) of cryopreservation the calculated amount of O_2^- was decreased, suggesting a loss of stability of cryopreserved reduced cyC. Additional control experiments investigating the stability of non-reduced cell-free cyC kept frozen in the assay mixture did not show any alterations when the solution was stored at –20 °C and thawed after 2, 8 or 16 weeks [optical density, means (SEM) T0 = 0.150 (0.011), T1 = 0.152 (0.016), T2 = 0.152 (0.013), T3 = 0.154 (0.019); $n = 11$].

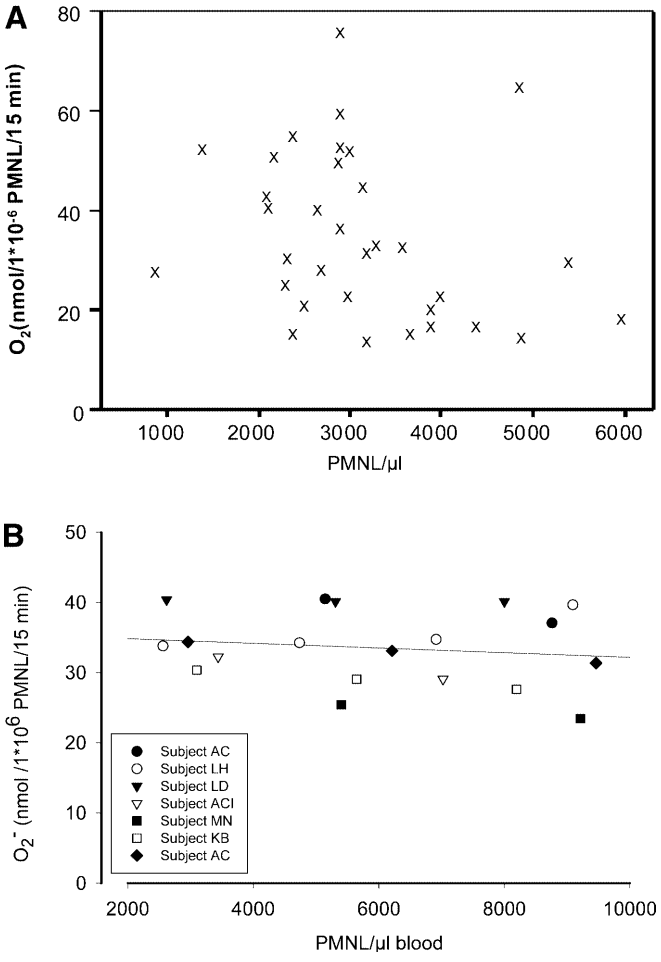

Fig. 2. **A** Relationship between normally distributed numbers of polymorphonuclear leucocytes (*PMNL*) per microlitre of whole blood and the rate of N-formyl-methionyl-leucyl-phenylalanine (*fMLP*)-stimulated superoxide anion (O_2^-) production. From the blood of healthy young volunteers PMNL counts were determined and compared to the individual fMLP (10^{-6} mol/l) stimulated O_2^- production rate. In addition to the high interindividual variability in the production of O_2^- at the same PMNL concentration there was no correlation between the number of PMNL/µl and the O_2^--production-rate (each individual is represented by a *cross*). Means (SEM), volunteers' age = 28.45 (1.35) years, O_2^- production-rate = 33.75 (16.6) nmol/1*10^{-6} PMNL/15 min, PMNL/µl = 3260 (1113); r (Pearson correlation coefficient) = –0.21, P (two-tailed level of significance) = 0.22; $n = 33$. **B** Effect of increasing PMNL number (less than 10000/µl) on the fMLP-stimulated O_2^- production. To investigate the reliability of the whole-blood assay at PMNL concentrations higher than those observed in healthy volunteers, shown in **A**, PMNL concentrations were further increased by the addition of separated cells to the whole-blood assay. The fMLP-stimulated rate of O_2^- production (nmol/1*10^6PMNL/15 min, fMLP 10^6 mol/l) remained constant up to 10000 PMNL/µl. [Seven different individuals, each individual is represented by his initials, volunteers' mean (SEM) age = 31 (1.8); linear regression analysis between O_2^- production and PMNL/µl less than 10,000, $r = 0.15$; $n = 19$]

Effects of storage time on leucocyte and granulocyte counts

To answer the question as to whether leucocyte concentrations in blood samples anticoagulated with EDTA

Table 2. Effect of polymorphonuclear leucocyte (*PMNL*)-concentration (more than 10,000·µl^{-1}) on the N-formyl-methionyl-leucyl-phenylalanine (*fMLP*)-stimulated superoxide anion (O_2^-) production. To investigate the reliability of the whole-blood assay at PMNL concentrations higher than 10,000 µl^{-1}, cell concentrations were increased by the addition of previously separated PMNL. In all subjects the fMLP-stimulated rate of superoxide anion production (nmol·l^{-1}×10^6 PMNL·15 min^{-1}, fMLP 10^6 mol·l^{-1}) decreased when the PMNL concentration was greater than 1×10^4·µl^{-1}. Seven different individuals, each individual is represented by his initials. Age of volunteers [mean (SEM)] = 31 (1.8) - years; linear regression analyses between O_2^- production and PMNL·µl^{-1} greater than 10,000·µl^{-1} gave a coefficient of correlation *r* of 0.77, $P < 0.05$; $n = 22$

Subjects	PMNL·µl^{-1}	fMLP(10^{-6} mol·l^{-1})-stimulated O_2^- production (nmol·l^{-1}×10^6 PMNL)
AC	11,267	31.59
	13,444	26.00
LH	12,375	29.59
	15,989	23.67
	23,218	17.35
LDS	10,692	35.21
	10,692	35.21
	13,383	27.51
	16,074	14.68
MT	11,204	20.99
	15,064	13.06
	18,919	11.80
	22,774	6.77
ACI	10,605	23.49
	17,771	14.96
	21,353	14.29
KB	10,741	22.74
	13,288	19.11
	15,836	15.52
ACII	12,710	22.11
	15,960	17.58
	19,210	13.98

and kept at room temperature would change over time (e.g. due to transportation), the absolute leucocyte concentrations and automated differentiation of PMNL were assessed after 8, 16, 24, 48, 72 or 96 h and compared to those obtained with immediate determination (Table 3). The results indicate that automated counts can reliably be achieved at least up to 24 or 48 h, respectively. When granulocyte counts are measured 96 h after blood withdrawal, the measurements appear not to be correct.

Interference of free haemoglobin with photometry

There was no free haemoglobin detectable in assays run in the absence of cyC (data not shown).

Assay reliability

To evaluate the assay precision, the intra- and inter-assay coefficients of variation were determined. The values were 16% and 14%, respectively, and remain in the range of other biochemical tests routinely used for clinical diagnosis (Thomas 1992).

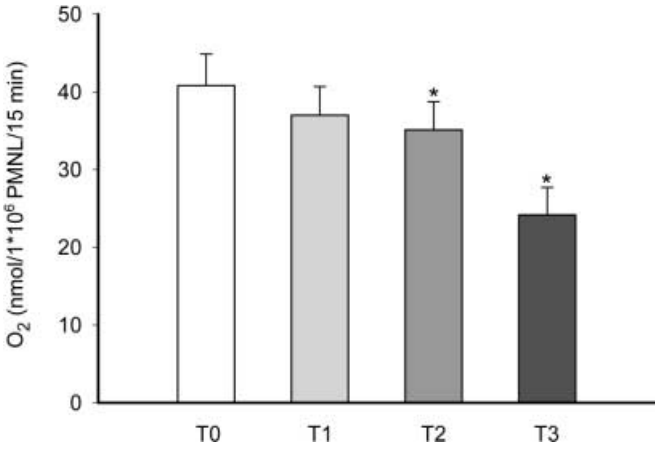

Fig. 3. Long-term cryopreservation. After stimulation of diluted blood specimens using N-formyl-methionyl-leucyl-phenylalanine (10^{-6} mol/l) cell free supernatants were removed from the whole-blood assays and split into four portions. Three of them were frozen at –20°C and thawed after 2 (*T1*), 8 (*T2*) or 16 (*T3*) weeks. The values measured at T1 did not differ significantly from those determined immediately before freezing (*T0*), whereas at T2 they just exceeded the level of significance.. No significant changes were observed between mean values at T1 and T2. [means (SEM) volunteers' age = 31.45 (1.71) years, multiple comparison by Bonferroni *t*-test, $*P < 0.05$, lower/upper 95% confidence interval for the mean difference, T0 compared to T1 = 0.83/7.41, T0 compared to T2 = 2.44/8,85, T0 compared to T3 = 14.24/25.68; $n = 11$]

Discussion

Evaluation of the whole-blood assay

The PMNL and monocytes generate reactive O_2^-. Thereafter, O_2^- can subsequently form other highly reactive metabolites (hydroxyl radicals, hypochloric acid, hydrogen peroxide) which play an important role in the host defence against a large spectrum of micro-organisms (for review see Yang and Hill 1991). Moreover, O_2^--derived reactive oxygen metabolites can greatly contribute to tissue damage during inflammation. Because of the pathogenetic role that O_2^- play in the aforementioned processes, various methods have been developed to detect the extracellular release of these reactive oxygen metabolites from phagocytes. Among them, the most commonly used methods are based either on lucigenin enhanced chemiluminescence (Root and Metcalf 1977; Vasquez Vivar et al. 1997) or on photometric determination of the reduction of ferricytochrome C (Markert et al. 1984). The latter method, the SOD-inhibitable reduction of cyC, is the only one which allows the specific quantification of the extracellular release of O_2^- (Arthur et al. 1987). On the other hand this technique usually requires the isolation of leucocytes from whole blood. Despite standardization of cell-isolation procedures, the agents and conditions, e.g.

Table 3. Effect of storage on leucocyte and granulocyte counts delayed determination of blood cell counts of leucocytes, granulocytes and monocytes. Total leucocyte and granulocyte blood counts were performed either immediately (0 h) or after 8, 16, 24, 48, 72 and 96 h of storage at room temperature using a Coulter STKS counter. Mean (SEM) volunteers'age = 31.45 (1.71) years, $n = 5$]

Time		Cell-counts	
		Leucocytes·µl^{-1}	PMNL·µl^{-1}
0 h	Mean	5,760	3,290
	SEM	1,620	120
8 h	Mean	5,740	3,240
	SEM	1,640	1,300
16 h	Mean	5,700	3,220
	SEM	1,830	1,410
24 h	Mean	5,360	3,030
	SEM	1,570	1,240
48 h	Mean	5,360*	3,250
	SEM	1,500	1,180
72 h	Mean	5,480	3,370
	SEM	1,500	1,220
96 h	Mean	5,230*	2,990
	SEM	1,460	240

*Multiple comparison by Bonnferoni's *t*-test $P < 0.05$ compared to 0 h

dextran-sedimentation, Ficoll or Percoll gradient sedimentation and hypotonic lysis of remaining RBC, may all contribute to unpredictable cell changes and/or preactivation (Hamblin et al. 1992; Richter 1992). Activation of PMNL merely by separation was shown in our experimental setting by the increased expression of $\beta 2$-integrins on isolated PMNL and increased rates of fMLP-stimulated O_2^- production compared to granulocytes in diluted whole blood. In addition, isolation of PMNL appeared to render cells into a primed state, because very low concentrations of fMLP (10^{-9} mol·l^{-1}) induced the production of substantial quantities of O_2^- whereas granulocytes in diluted whole blood did not show any response.

With respect to these limitations, we looked for the possibility of determining the production of O_2^- in a whole-blood assay thereby removing the need for cell isolation. For this purpose cyC was added to heparinized whole blood. Following phagocyte stimulation by the chemotactic tripeptide fMLP for 15 min at 37°C, cellular elements were centrifuged and the amount of reduced cyC was determined in the supernatants by photometry. Based on the molar extinction coefficient the formation of O_2^- was calculated as nanomoles of O_2^- produced per 1,000,000 phagocytes, i.e. predominantly by neutrophils.

A prerequisite of this assay was the determination of the O_2^- production rate irrespective of the number of PMNL in the assay. This was demonstrated in a large number of healthy volunteers by the lack of any correlation between the normally distributed granulocyte counts and the rate of O_2^- production per 1,000,000 PMNL (Fig. 2A). However, the granulocyte concentrations used for this calculation were in the normal physiological range and therefore did not exceed a maximal concentration of 6,000 PMNL·µl^{-1}. The only way of demonstrating unaltered O_2^--rates at higher PMNL concentrations was by adding previously separated granulocytes to the whole-blood assay. For that purpose granulocytes were isolated from whole blood and added thereafter to the whole-blood assays made on the blood from the same donor. We decided to use these separated cells *only* for testing a possible relationship between higher numbers of granulocytes and O_2^--production in the whole-blood assay, being aware of the aforementioned preactivation and higher rates of O_2^--production of isolated PMNL. The results of these experiments demonstrated that the production rate of O_2^- remained constant if the concentration of PMNL did not exceed 1×10^4 PMNL·µl^{-1} in the whole blood assay (Fig. 2B). Increasing the PMNL concentration above this limit caused a decrease in the O_2^- production-rate calculated per 1,000,000 PMNL (Table 2).

This might have been due to the exhaustive reduction of cyC. The final concentration of 50 µmol·l^{-1} cyC would be sufficient to react with 83 nmol of O_2^- in the assay mixtures. Given a final concentration of 11,000 PMNL·µl^{-1} whole blood and a blood volume of 250 µl diluted in the final assay volume, the total number of PMNL was calculated to be 2.75×10^6 PMNL. By multiplying the averaged rate of 30–35 nmol·10^6 PMNL·15 min^{-1} by the 2.75×10^6 PMNL gives an averaged amount of O_2^- of 83–96 nmol which could completely reduce the cyC present in the assay mixture. Control experiments revealed that the exhaustion of cyC reduction could be partly overcome by the addition of further cyC. When the final cyC concentration was doubled (100 µmol·l^{-1}) the calculated production rate increased from 13.8 to 29.6 nmol O_2^-·10^6PMNL·15 min^{-1} (mean values: fMLP 10^{-6}mol·l^{-1}, volunteer age = 31.8 years, PMNL concentration 1.4×10^4·µl^{-1}; $n = 5$).

Thus, when the need occurs to determine the production of O_2^- at blood concentrations of phagocytes exceeding 1×10^4·µl^{-1} an increase in the concentrations of reducable cyC in the assay might be useful. However, the presence of artefacts and the limitations of spectophotometric measurements should always be taken into consideration because the light extinction increases by a further addition of cyC to the assay.

Regarding the cell type predominantly involved in the production of O_2^- in the whole-blood assay, the relationship between the fMLP-mediated O_2^- production in whole blood and isolated PMNL first suggest that O_2^- production was mainly due to stimulation of PMNL. However, when blood is obtained from patients having an allergic disease (e.g. allergic bronchial asthma, or hay fever) or a parasite-induced disease, the percentages of eosinophils cannot differentiate between O_2^- production by neutrophils from that by eosinophils. In contrast, monocytes are unlikely to contribute significantly to fMLP-stimulated O_2^- production, because of the low percentage of circulating monocytes (4%, $0.2–0.6\times10^3$·µl^{-1} whole blood), and their slow kinetic in

producing O_2^- (Bellavite et al. 1983; Bielefeldt and Babiuk 1984), reaching maximal production rates with potent activators as long as 60 min after their addition. Accordingly, in the studies published on the determination of O_2^- in whole blood by the reduction of cyC, the role of monocytes has not been addressed so far. Moreover, in these studies phagocytes were completely activated either by opsonized zymosan particles or by the non-physiological activator phorbol myristate acetate (PMA) (Bellavite et al. 1983). The PMA is a very strong stimulus, which, in contrast to fMLP, bypasses the receptor-dependent signal transduction, leading, by the direct activation of the protein kinase C, to a maximal response. Although activation of phagocytes by opsonized zymosan particles represents stimulation of cells in a physiological way, activation by PMA may be considered to be an unphysiological process. In our experiments the chemotactic tripeptide fMLP-induced production of O_2^- was characterized in whole blood and isolated PMNL. The latter is of clinical relevance, because activation by fMLP may represent the biological effects of bacterial peptides with well-known chemotactic and activating properties. In our whole blood assay as well as when using isolated PMNL, we observed under both conditions a fMLP dose-dependent, up to a sixfold, increase in the production of O_2^- (Fig. 1). The values obtained were in the range of reported values elicited by fMLP in isolated neutrophils (Grzeskowiak et al. 1986; Wilson et al. 1986), which supports the reliability of the assay. Moreover, the intra- and inter-assay coefficients of variation were in the same acceptable ranges as other biochemical tests used routinely for clinical diagnosis (Thomas 1992). The rather high variability illustrated by the SEM, however, could have been due to interindividual differences of the oxidative metabolism (Fig. 2A) which can be modulated by age and sex. To reduce this source of error only young (mean age 31 years) men and healthy subjects were included in the study. However lifestyle, individual nutrition conditions and other factors which might have affected the results (Bellavite et al. 1995) could not be detected in this study. Finally, despite the fact that we used a whole-blood assay, cells were activated by the physiological soluble chemotactic tripeptide fMLP.

Cryopreservation of reduced cyC

Hence, the whole-blood assay seems to be practicable for the study of changes in the cellular activity of PMNL under clinical conditions. In addition, we considered the possibility of whether reduced cyC can be frozen and determined by photometry at a later time after blood processing. The results indicated that reduced cyC could be stored at only –20°C for at least 2 weeks without any significant change in the quantities of O_2^- calculated. However, when O_2^- production was determined after 8 weeks of cryopreservation, results were significantly lower compared to immediate determination without freezing (Fig. 3). Although there was a decrease in the calculated rate of O_2^- production by 10%–20% after cryopreservation, this decline may not exclude the evaluation of changes in the oxidative capacity of granulocytes, e.g. between different treatment groups or experimental conditions even 8 weeks after blood sampling and cryopreservation at –20°C. The use of samples stored for more than 8 weeks seems not to be appropriate because of major reduction of the O_2^- production rates after 16 weeks of cryopreservation. The causes of these time-dependent changes in the absorbances of cryopreserved samples are unclear. One is tempted to speculate about complex interactions between blood components and reduced cyC, because the cyC-containing reaction mixture which was cryopreserved without prior incubation with cells remained unaffected by similar freezing periods.

Freezing of supernatants may not be required in clinical trials which normally take place in research centres and hospitals where a highly developed infrastructure can be shared and O_2^- production can be determined almost on-line, e.g. by sophisticated techniques (chemiluminescence, flow cytometry). In contrast, when investigations have to be performed in surroundings which do not provide such methods, the determination of the production of O_2^- can be achieved by the present whole blood assay with the help of simple devices (heated water or thermal block, manually driven centrifuge, cold packs). After blood processing, supernatants can be frozen and kept refrigerated until further analysis. Following the thawing of the samples, the more elaborate photometric determination of cryopreserved cyC can be performed up to 2 weeks later. Since the practicability and scientific value of this method is based on the correct determination of the number of PMNL (e.g. nmol $O_2^- \cdot l^{-1} \times 10^6$ PMNL) it is was important to test the effects of storage time on (e.g. for transportation) PMNL counts in blood samples. From our results and experience the following suggestions can be given: when the determination of blood cell counts is possible within 48 h following blood acquisition, PMNL concentrations can be determined from EDTA-anticoagulated blood by automated cell counters (Coulter STKS). Between 3–7 days, reliable leucocyte counts are still possible using a Coulter STKS [recommendations of the German Asssociation for Clinical Chemistry (2000), Bonn, Germany], but blood smears should be performed after blood sampling to calculate the absolute PMNL concentration from the percentage of PMNL identified on the smears. Dried but not further prepared smears can be stored up to 4 weeks before dyeing (May-Grünwald and Giemsa).

The value of this assay has already been demonstrated in studies investigating the effects of acute and chronic stress on granulocyte functions and on the innate immune system during simulated microgravitation (Choukèr et al. 2001), confinement experiments (Choukèr et al. 2002) and mountain medicine at high altitude (Choukèr et al. 2000). Additionally, this method

has been considered for use on the International Space Station [ISS].

Taken together, the results from the present study have shown how a widely used method for detecting O_2^- could be improved to allow detection of O_2^- in whole-blood assays under more physiological conditions, while eliminating the influence of separation procedures and minimizing the sample volume required. Numbers of PMNL up to 10,000 µl did not affect the ability of the cyC assay to determine O_2^- production reliably. Moreover long-term cryopreservation at least up to 2 weeks allowed determination of stimulated O_2^- production at later times. This may turn out to be of great advantage when the oxidative metabolism of phagocytes is to be studied under extreme enviromental or field conditions in, for example, space and mountain medicine.

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