Intensive Care Med (1997) 23: 743–748
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ORIGINAL

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A controlled study of leukocyte activation in septic patients

Received: 2 December 1996
Accepted: 14 May 1997

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Abstract *Objective:* Qualitative and quantitative evaluation of leukocyte activation in septic patients in comparison to two control groups. *Design:* A prospective clinical study in which the leukocyte oxidative output of whole blood was measured in three groups of patients. Two chemiluminescence markers (luminol or lucigenin), indicative of either total oxidant output or superoxide production, and three stimuli (opsonized zymosan, formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate) (PMA), representing different pathways of leukocyte activation, were used. Tumor necrosis factor, interleukin-6 and C-reactive protein (TNF, IL-6, and CRP) were determined to evaluate the severity of the inflammatory process.
Setting: Intensive care and surgical units of a university hospital.
Patients: Seventy-four healthy patients, ten ICU patients without signs of sepsis or systemic inflammatory response syndrome and 19 septic patients were studied.
Measurement and main results: With all three stimuli, whole blood total oxidative output and superoxide production were generally increased in septic patients. This was most likely due to the increased leukocyte numbers in these patients. When the chemiluminescence values were normalized per phagocyte (granulocytes and monocytes), the total oxidative output of septic phagocytes decreased with opsonin and fMLP but increased with PMA, while superoxide output decreased regardless of the stimuli used. TNF, IL-6 and CRP, although increased in septic patients as compared to ICU controls, correlated weakly with oxidant output.
Conclusions: The oxidative output of whole blood was increased in septic patients compared to controls because of elevated leukocyte numbers. However, oxidant output normalized for phagocyte numbers generally decreases during sepsis for most stimuli. Cytokines and CRP do not appear to be associated with the extent of oxidant output during sepsis.

Key words Sepsis · Septic shock · Respiratory burst · Oxidative burst · Leukocytes · Chemiluminescence · Phorbol myristate acetate · Opsonized zymosan · Formyl-methionyl-leucyl-phenylalanine

Introduction

Leukocyte activation, characterized by the respiratory burst, is an essential part of host defense against invading microorganisms [1, 2]. The respiratory burst leads to the formation of toxic oxidant through NADPH oxidase, an enzyme with a complex structure and activation process [3]. Bacterial products, opsonized material and unusual surfaces are all possible stimuli of leukocyte activation and toxic oxidant production [3, 4]. Since toxic

Table 1 Patient characteristics

	Healthy controls	ICU controls	Septic patients
Numbers	74	10	19
Age	44 (20–74)	46 (20–78)	54 (19–75)
Gender (male/female)	20/54	7/3	12/9
APACHE-score*	–	35 (20–44)	38 (29–44)
Survival (in %)	100	90	32

Median (range). * sedated patients were all assigned a Glasgow Coma Scale value of 3

oxidants can not only be directed to kill microorganisms but may also damage host tissues, the process of leukocyte activation is strictly regulated [2]. Several cytokines have been shown to augment the response of leukocytes to subsequent activation [3]. Excessive cytokine response in the course of systemic inflammation and sepsis may therefore lead to an exaggerated leukocyte response to stimuli and increased production of toxic oxidants, thus contributing to tissue damage and organ injury.

Several investigators have reported activation of leukocytes during sepsis [4–6]. However, many studies are uncontrolled or only include healthy controls. Inclusion of only healthy controls does not take into account that even non-septic ICU patients may present with differences in leukocyte activation. Furthermore, differences in biochemical methodology (differences in the activating stimuli used, differences in the oxidants measured, measurements in whole blood or in leukocyte suspensions) make it difficult to compare results and draw valid conclusions. Better understanding of leukocyte activation during sepsis may help identify and select patients who could potentially benefit from novel pro- or anti-inflammatory therapies.

In this study we describe leukocyte activation in septic patients in comparison with two control groups: patients without sepsis or inflammatory response syndrome (SIRS) and minor surgery patients not on the ICU. To study the quality of the oxidative response we used two chemiluminescence markers – luminol, indicative of both intracellular and extracellular total oxidant production, and lucigenin, indicative of extracellular superoxide production [7]. Furthermore, we used both receptor-associated and non-receptor-associated stimuli to activate the respiratory burst. To quantify the inflammatory response, we measured C-reactive protein (CRP), tumor necrosis factor alpha (TNF) and interleukin-6 (IL-6).

Methods

Patients (Table 1)

One hundred three patients of a university hospital were included in this study. The study was approved by the institutional Ethical Committee and informed consent was obtained from the patient or his/her legal representative.

Seventy-four patients awaiting minor surgery with no signs of systemic disease or infection which might affect leukocyte function served as healthy controls; ten ICU patients without sepsis or SIRS served as ICU controls and 19 septic patients comprised the study group. The diagnostic criteria for sepsis were applied as defined by a recent consensus conference on sepsis [8]. Patients receiving immunomodulatory drugs (steroids, filgrastim, anti-cytokine therapy) were not included in this study. The patients were studied on the first and subsequent days during which their clinical presentation met the diagnostic criteria of sepsis. A clinician not involved in the study classified the patients on the ICU.

Materials

Phosphate-buffered saline with glucose and endotoxin-free albumin (PBSGA: pH = 7.3; 4.58 mM KH_2PO_4, 8.03 mM Na_2HPO_4; 0.8 %[w/v] NaCl, 0.02 %[w/v] KCl, 0.1 %[w/v]glucose and 0.1[w/v] endotoxin-free albumin was used). The standard buffer consisted of pH = 7.3; 4.58 mM KH_2PO_4, 8.03 mM Na_2HPO_4; 0.76 %[w/v] NaCl, 0.033 %[w/v] KCl, 0.1 %[w/v] glucose, 0.1[w/v] endotoxin-free albumin, 0.5 mM $MgCl_2$ and 0.45 mM $CaCl_2$). The phorbol myristate acetate (PMA), opsonized zymosan (OPSONIN), formyl-methionyl-leucyl-phenylalanine (fMLP), lucigenin and luminol were all obtained from Sigma (Deisenhofen, Germany).

Assays

A whole blood assay was used because cell separation procedures may in themselves cause activation or priming of leukocytes [9]. Leukocyte activation was evaluated by two separate markers: 1) luminol chemiluminescence as an indicator of total intracellular and extracellular oxidant production and 2) lucigenin chemiluminescence as an indicator of superoxide production. The reaction for chemiluminescence was performed as described previously [10] with minor modifications. The procedure was adapted to a microtitre plate format with a 96 well luminometer (Berthold MicroLumat LB 96, Bad-Wildbad, Germany). Whole arterial blood was collected anaerobically in EDTA-K tubes and assayed immediately. Fifty microlitres of whole blood was diluted in 5 ml PBSGA. For luminol determination, 50 µl of diluted blood was added to 1.1 ml of standard buffer containing 100 µl luminol 3.5 mM. For lucigenin determination, 100 µl of diluted blood was added to 1.025 ml standard buffer containing 25 µl lucigenin 10 mM.

Three different types of stimuli corresponding to three different activation pathways of the respiratory burst were used: fMLP representing receptor-dependent stimulation by peptides of bacterial origin [4]; opsonin representing receptor-dependent stimulation by opsonized material and PMA representing receptor-independent stimulation [4, 11]. Fifty microlitres of either standard buffer, fMLP, opsonin or PMA were added to 150 µl of dilute whole blood containing either lucigenin or luminol. The probes were in-

cubated for 65 min at 37 °C, the maximal light emitted during this period was measured and the results expressed in Relative Light Units per second ($RLU.s^{-1}$). Leukocyte counts were determined with an automated cell counter and the chemiluminescence values were then normalized and expressed in $RLU.s^{-1}$ per phagocyte. Non-normalized values are presented as $RLU.s^{-1}$ per well (corresponding to 0.065 µl and 0.13 µl whole blood per well for luminol and lucigenin, respectively). Tumor necrosis factor alpha (TNF) and interleukin-6 (IL-6) were measured (both Medgenics Diagnostics SA, Fleurus, Belgium) in the blood samples of all ICU patients (but not healthy controls). CRP was measured using the N-Latex CRP testkit (Behring-Werke, Marburg, Germany). Cytokine, CRP and chemiluminescence measurements were made on the same day.

Statistical analysis

Using SPSS for Windows (release 6.01), the chemiluminescence values for each stimulus were compared in the three study groups with a Kruskal-Wallis test and corrected for multiple comparisons (Bonferroni). Cytokine values (in two groups) were compared using a Mann-Whitney-test. Correlation coefficients (Spearman) were calculated between cytokines (TNF and IL-6) or CRP and chemiluminescence values. Significance was accepted at p less than 0.05. The results are expressed as the mean ± SEM or median (range) when apropriate.

Results

Figure 1 shows non-normalized values (not normalized per phagocyte) of chemiluminescence stratified by the type of stimulus, type of chemiluminescence and group of patients. Both luminol chemiluminescence, indicative of total oxidant production, and lucigenin chemiluminescence, indicative of superoxide production, were increased in septic patients as compared to the two control groups. The ICU controls did not differ from healthy controls. However, septic patients had significantly increased numbers of leukocytes (especially neutrophils) as compared to the other two groups (Table 2). We therefore normalized the chemiluminescence values for cells with oxidative capabilities (phagocytes: granulocytes and monocytes) and found that the ability of phagocytes to be activated and produce oxidants (as measured with luminol and lucigenin) was decreased with most stimuli. The only exception was luminol chemiluminescence with PMA as a stimulus, which showed an increase as compared to the other two groups (Fig. 2).

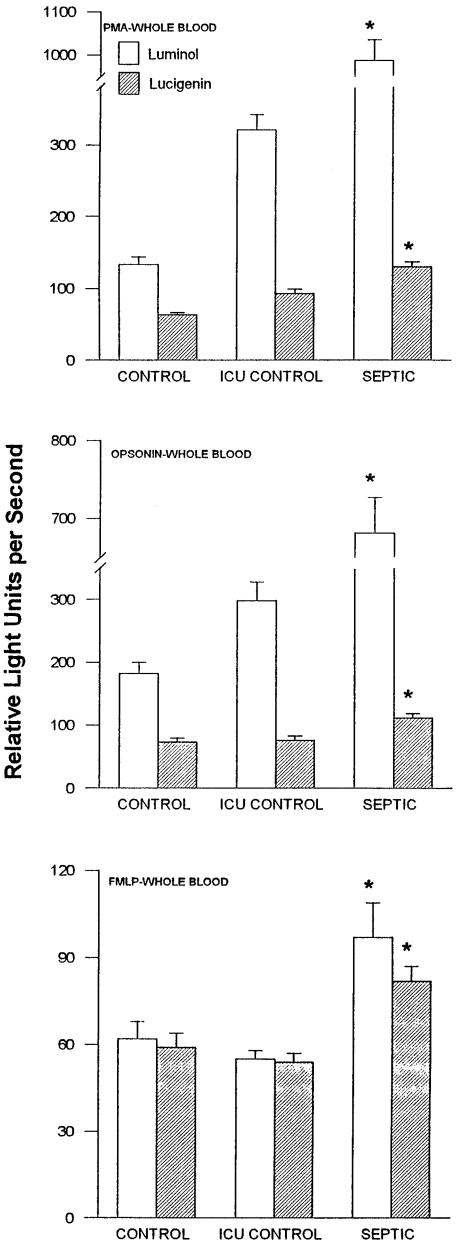

Fig. 1 Oxidant production capability of whole blood measured with two chemiluminescence markers (luminol and lucigenin) and three stimuli (*PMA* phorbol myristate acetate, *OPSONIN* opsonized zymosan, *FMLP* formyl-methionyl-leucyl-phenylalanine) in three groups of patients. *$p < 0.05$ as compared to the other groups

Table 2 Total leukocyte counts and differentials in the three groups of patients

Groups	Total leukocytes	% Neutrophils	% Lymphocytes	% Monocytes	% Others
Healthy controls	6.3 ± 0.3	61 ± 1	32 ± 1	4 ± 0.2	2 ± 0.2
ICU controls	8.5 ± 0.8	78 ± 4*	17 ± 2*	3 ± 0.9	2 ± 1.0
Septic patients	21.3 ± 1.0*#	87 ± 1*#	9 ± 2*#	2 ± 0.5	2 ± 0.5

Means ± SEM; *, # $p < 0.05$ compared to healthy controls and ICU controls, respectively

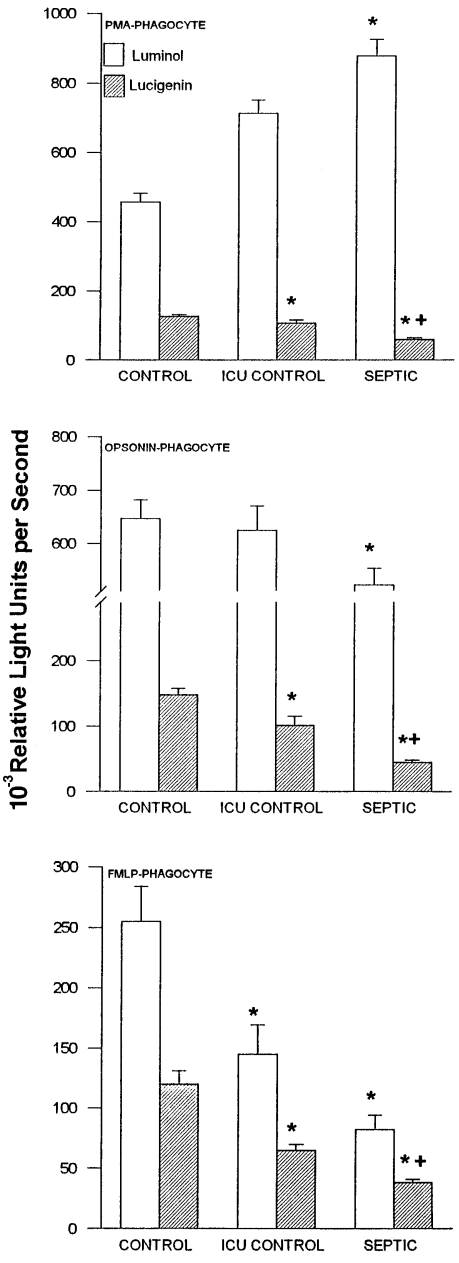

Fig. 2 Oxidant production capability per phagocyte measured with two chemiluminescence markers (luminol and lucigenin) and three stimuli (*PMA* phorbol myristate acetate, *OPSONIN* opsonized zymosan, *FMLP* formyl-methionyl-leucyl-phenylalanine) in three groups of patients. $*p < 0.05$ as compared to the control group, $+p < 0.05$ as compared to ICU controls

Of note is the fact that, depending on the stimulus and quality of oxidants, phagocytes from ICU patients without sepsis or SIRS also had some impairment in their ability to produce oxidants upon stimulation (Fig. 2). However, the whole blood oxidant output upon stimulation of these patients was not different from that of healthy controls (Fig. 1). TNF and IL-6 levels were significantly increased in septic patients as compared to ICU controls (Table 3). However, the correlation between oxidant output (whole blood or normalized per phagocyte) and cytokine values was low ($r < 0.4$; p = ns to < 0.01). CRP was increased in septic patients as compared to ICU controls.

Table 3 TNF, IL-6, and CRP levels in ICU controls and septic patients. (*TNF* tumor necrosis factor, *IL-6* interleukin-6, *CRP* C-reactive protein)

	ICU controls	Septic patients	p
IL-6 (pg/ml)	118 ± 67	934 ± 55	< 0.01
TNF (pg/ml)	15.6 ± 1.8	37.6 ± 2.6	< 0.01
CRP (mg/l)	67 ± 39	230 ± 123	< 0.01

Means ± SEM

Discussion

When stimulated with three different stimuli, whole blood oxidant output, measured with lucigenin and luminol chemiluminescence, was increased in septic patients as compared to the two control groups. However, this increase was most probably due to the increased numbers of phagocytes (and especially neutrophils) present in septic patients as compared to the other two groups. Normalizing oxidative output for individual phagocytes revealed that oxidative output upon stimulation with most stimuli was actually decreased in septic patients as compared to the other two groups. The PMA stimulus seems to be an exception as the total (luminol) oxidant output per phagocyte of septic patients stimulated with PMA still showed an increase compared to that of healthy controls.

In severe inflammatory states such as sepsis, both increases and decreases in the oxidant output of leukocytes in response to certain stimuli have been described. Trautinger, using PMA as a stimulus, reported an increase in peroxide production by the leukocytes of septic patients as compared to non-septics [6]. Tschaikowsky, using fMLP as a stimulus, reported an increase in superoxide production in septic and post-traumatic patients. However, using PMA as a stimulus, neutrophils from septic patients released less superoxide than those of normal controls [4]. Neutrophil dysfunction has been reported to occur in patients with acute bronchopneumonia [12], in patients with postoperative infections [13] and in patients with intra-abdominal sepsis [14]. Superoxide generation was found to be decreased in septic patients as compared to non-septic ICU controls [15]. These studies indicate that, to avoid conflicting results and misleading conclusions, oxidant output in severe inflammatory states such as sepsis should be viewed more

differentially. Inflammatory processes may lead to both qualitative and quantitative impairment in leukocyte activation, which can be better appreciated when these are studied together. In this study, we found a decrease in oxidative output for individual neutrophils during sepsis for most stimuli, suggestive of a quantitative impairment in leukocyte function during sepsis. However, PMA stimulation produced more luminol chemiluminescence (suggestive of greater total oxidant output) in neutrophils from septic patients, suggesting a qualitative impairment in leukocyte function during the disease process. Thus leukocyte function during inflammatory/septic disease may be impaired both quantitatively and qualitatively in a complex manner.

Why leukocyte function is impaired during SIRS and sepsis is not known. One possible explanation may be that exposure of the leukocytes to endogenous and exogenous mediators released during these diseases renders them less responsive to subsequent stimuli. Down-regulation of fMLP-receptors has been reported to occur in septic patients [4]. Cytokines (TNF and IL-6) and CRP levels were significantly increased in septic patients as compared to ICU controls, suggesting an increased inflammatory activity in these patients. We may assume that healthy controls had very low or non-detectable levels of these cytokines or of CRP. TNF is known to prime and activate the neutrophil [16] and IL-6 has been proposed as a measure of the inflammatory activity or severity of sepsis [17]. Despite this evidence of increased inflammatory activity, oxidant production of the individual phagocyte, as induced with various agents, was impaired in sepsis. This may be caused by receptor down-regulation, impairments in signal transduction or the predominance of other anti-inflammatory or antioxidant mechanisms during sepsis.

The different responses of the leukocytes to the three stimuli used (fMLP, opsonin, PMA) are probably related to their different activating pathways. fMLP, an oligopeptide of bacterial origin, binds to specific receptors on the neutrophil and activates many varied functions such as chemotaxis, aggregation and reactive oxidant production [4, 9, 18]. Opsonized material, too, interacts with neutrophils through specific receptors [19]. PMA is a potent activator of neutrophil oxidase and causes receptor-independent induction of the respiratory burst by directly activating protein kinase C. These different, albeit overlapping, pathways of activation may be differentially impaired during the inflammatory process, which must be taken into account when interpreting the respiratory burst during the disease process.

To investigate whether oxidant output is also qualitatively impaired during inflammatory/septic processes, we used two different chemiluminescence mechanisms to measure the type of oxidant produced. Although luminol and lucigenin detection of specific oxidant species may overlap, the differences measured most probably do reflect differences in the oxidant species produced. Luminol chemiluminescence is indicative of total intracellular and extracellular oxidant output by the leukocytes: various radicals and oxidative agents generated by NADPH and myeloperoxidase during phagocytosis [7, 20, 21]. Lucigenin chemiluminescence is more indicative of superoxide output by the leukocyte [7]. Luminol detects oxidants only in the presence of myeloperoxidase, whereas lucigenin chemiluminescence does not require this enzyme. Luminol, but not lucigenin, has the ability to permeate the cell and therefore also measures intracellular oxidants. Thus differences in luminol and lucigenin measurements with the PMA stimulus may reflect qualitative changes in the oxidants produced.

Besides healthy patients, we also included ICU patients without sepsis as controls in order to determine the effects from sepsis independent of other factors common to ICU patients. Major operations, trauma, invasive monitoring and ICU stay may all contribute to phagocyte dysfunction. Our study shows that some aspects of their phagocyte oxidative capabilities were indeed impaired in these patients. The phagocyte oxidative capabilities of septic patients, however, were overall more impaired than those of the ICU controls. This suggests that impairments in the oxidant production upon stimulation were specific to sepsis and not due to concurrent disease, medication or ICU stay as such.

In this study we used whole blood and normalized the values according to the phagocyte numbers. Using whole blood instead of separated neutrophils or other phagocytes for this purpose has some advantages. The separation process may lead to loss of activated or primed phagocytes, due to clumping or adhesion; thus, mainly unprimed phagocytes may survive, leading to results not reflecting the true oxidative capacity. In addition, the separation process may lead to activation of unprimed phagocytes or phagocyte function may change during the time-consuming separation procedure. Whole blood analysis of phagocyte function has proven to be a versatile tool in both experimental and clinical studies [22–24].

Although our findings suggest phagocyte dysfunction during sepsis, it is not clear whether agents capable of increasing leukocyte function (e. g. granulocyte colony-stimulating factor) will prove helpful in non-neutropenic septic patients. First, as our results indicate, the total oxidant production of whole blood may still be increased due to the increase in the total number of leukocytes present. Second, it is not clear whether the diminished oxidant output of phagocytes is the result or, at least in part, the cause of sepsis-associated morbidity and mortality. Lastly, it still remains difficult to judge the adequacy of the delicate pro- and anti-inflammatory balance in the individual patient.

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