

**FLOW CYTOMETRIC PARAMETERS OF  
NEUTROPHIL FUNCTION AS EARLY  
INDICATORS OF SEPSIS- OR  
TRAUMA-RELATED PULMONARY OR  
CARDIOVASCULAR ORGAN FAILURE**

**GREGOR ROTHE,  
WOLFGANG KELLERMANN,  
and  
GÜNTER VALET**

**Martinsried and Munich, Federal Republic of  
Germany**

**From the Mildred-Scheel-Labor für Krebszellforschung, Max-  
Planck-Institut für Biochemie, Martinsried, and Institut für  
Anästhesiologie der Ludwig-Maximilians-Universität,  
Klinikum Großhadern, Munich.**

**Reprinted from  
THE JOURNAL OF LABORATORY AND CLINICAL  
MEDICINE,  
St. Louis**

**Vol. 115, No. 1, pp. 52-61, Jan., 1990  
(Copyright © 1990, by The C.V. Mosby Company)  
(Printed in the U.S.A.)**

# Flow cytometric parameters of neutrophil function as early indicators of sepsis- or trauma-related pulmonary or cardiovascular organ failure

GREGOR ROTHE, WOLFGANG KELLERMANN, and GÜNTER VALET

MARTINSRIED AND MUNICH, FEDERAL REPUBLIC OF GERMANY

Flow cytometric parameters of neutrophil function, such as phagocytosis and degradation of *Escherichia coli*, intracellular pH value, esterase activity, and cell volume, were evaluated as risk indicators for sepsis- and trauma-related pulmonary and cardiovascular organ failure in intensive care patients. Serial blood samples ( $n = 201$ ) were obtained from 47 prospectively identified patients. Each patient's condition was classified daily within four categories: post-traumatic ( $n = 22$ ) or septic ( $n = 28$ ) organ failure, transition state ( $n = 119$ ), and stable organ function after recovery ( $n = 27$ ). Thirty-two parameters of neutrophil function were automatically calculated for each blood sample from several flow cytometric list mode measurements of cell samples vitally stained with acridine orange for intact and denatured DNA or with 1,4-diacetoxy-2,3-dicyanobenzene for intracellular pH and esterase activity. The DNA of dead cells was simultaneously counterstained with propidium iodide. The cell biochemical parameter pattern was significantly different among samples of patients from the four clinical categories ( $p < 0.05$ ). Hyperergic phagocytosis was observed after trauma, in contrast to hypoergic phagocytosis, increased neutrophil cell volume, and elevated intracellular pH during sepsis. The clinical categories were correctly identified in 82% of the samples by automated classification with the DIAGNOS1/SPSS program system from the flow cytometrically determined cell functions. The course of the disease was correctly predicted 3 days in advance to the clinical manifestation of pulmonary or cardiovascular organ failure in 92% of the samples. The multifunctional analysis of neutrophils by flow cytometry seems of interest for early medical intervention in pre-septic and pre-shock patients. (J LAB CLIN MED 1990;115:52-61)

**Abbreviations:** ADB = 1,4-diacetoxy-2,3-dicyanobenzene; AO = acridine orange; DCH = 2,3-dicyanohydroquinone; DMF = *N,N*-dimethylformamide; *E. coli* = *Escherichia coli*; HBS = 5-mmol/L HEPES-buffered saline (pH 7.35); ICU = intensive care unit; PI = propidium iodide

From the Mildred-Scheel-Labor für Krebszellforschung, Max-Planck-Institut für Biochemie, Martinsried, and Institut für Anästhesiologie der Ludwig-Maximilians-Universität, Klinikum Großhadern, Munich.

Supported in part by Deutsche Forschungsgemeinschaft Sonderforschungsbereich 0207, project G6.

Submitted for publication March 15, 1989; revision submitted Sept. 1, 1989; accepted Sept. 6, 1989.

Reprint requests: Prof. Dr. Günter Valet, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-8033 Martinsried, Federal Republic of Germany.

5/1/16708

**P**osttraumatic and septic multiple organ failure are intimately related to changes of neutrophil function. A decreased chemotactic and phagocytic activity of neutrophils leads to bacterial infection, whereas increased activation of neutrophils may induce microvascular endothelial damage.<sup>1</sup> The abundant pulmonary sequestration of neutrophils and the release of large quantities of proteolytic enzymes and reactive oxygen metabolites are associated with the development of the sepsis- and trauma-related adult respi-

ratory distress syndrome.<sup>2,3</sup> Neutrophil functions are altered during infection,<sup>4-7</sup> after thermal injury,<sup>8,9</sup> or after trauma,<sup>10,11</sup> and high neutrophil or band neutrophil counts in peripheral blood are well-known indicators of infectious complications in compromised patients.<sup>12</sup>

The functional analysis of neutrophils is not performed in the routine clinical laboratory because of the lack of fast, sensitive, and reproducible methods for the analysis of variations of neutrophil function.

We recently developed two multiparametric flow cytometric assays for the analysis of neutrophil function.<sup>13</sup> High reproducibility is achieved by minimal sample manipulation, standardized measurement, and automated data evaluation. Buffy coat leukocytes are incubated with viable *E. coli* K12 bacteria in autologous plasma. The phagocytosis and degradation of bacteria, the increase of neutrophil cell volume, and the extent of cell death during phagocytosis are measured after staining with AO and simultaneous counterstaining of dead cells with PI. The esterase activity and the intracellular pH of viable phagocytosing neutrophils are determined after simultaneous staining with ADB and PI.

The goal of this study was to evaluate the potential of these assays as indicators of risk and course of sepsis and trauma-related pulmonary and cardiovascular organ function in ICU patients.

## METHODS

**Patients.** Patients of an operative ICU with a high risk for pulmonary or cardiovascular organ failure were selected by two criteria: a severe trauma with a trauma score  $>15$ <sup>14</sup> or unstable pulmonary or cardiovascular organ function after complicated surgery. Patients with hematologic malignancies, chronic renal failure, liver disease, diabetes mellitus, or during immunosuppressive treatment were excluded from the study. After informed consent, blood samples were drawn serially in 24-hour or 48-hour intervals, until discharge to normal care or patient death.

Control blood samples ( $n = 24$ ) from 14 healthy individuals were analyzed in parallel to define the normal range.

Research with patients and control persons was carried out according to the principles of the declaration of Helsinki, with approval by the Human Studies Committee of the University of Munich.

**Leukocytes.** Buffy coat leukocytes were prepared from 5 ml of heparinized (10 U/ml) venous blood by sedimentation at 200 g and 4° C for 10 minutes. The leukocytes were collected at the erythrocyte/plasma interface and resuspended in the supernatant plasma at a final concentration of  $3.5 \times 10^7$  leukocytes/ml. The pH value of the blood plasma rose to pH 7.80 through loss of CO<sub>2</sub> during equilibration of the blood with the ambient air and was readjusted to pH 7.40 by addition of approximately 10  $\mu$ l of 1 N HCl per ml plasma.

**Bacteria.** Stationary cultures of *E. coli* K12 (Sigma Chemie, Deisenhofen, FRG) were obtained after 2 days of

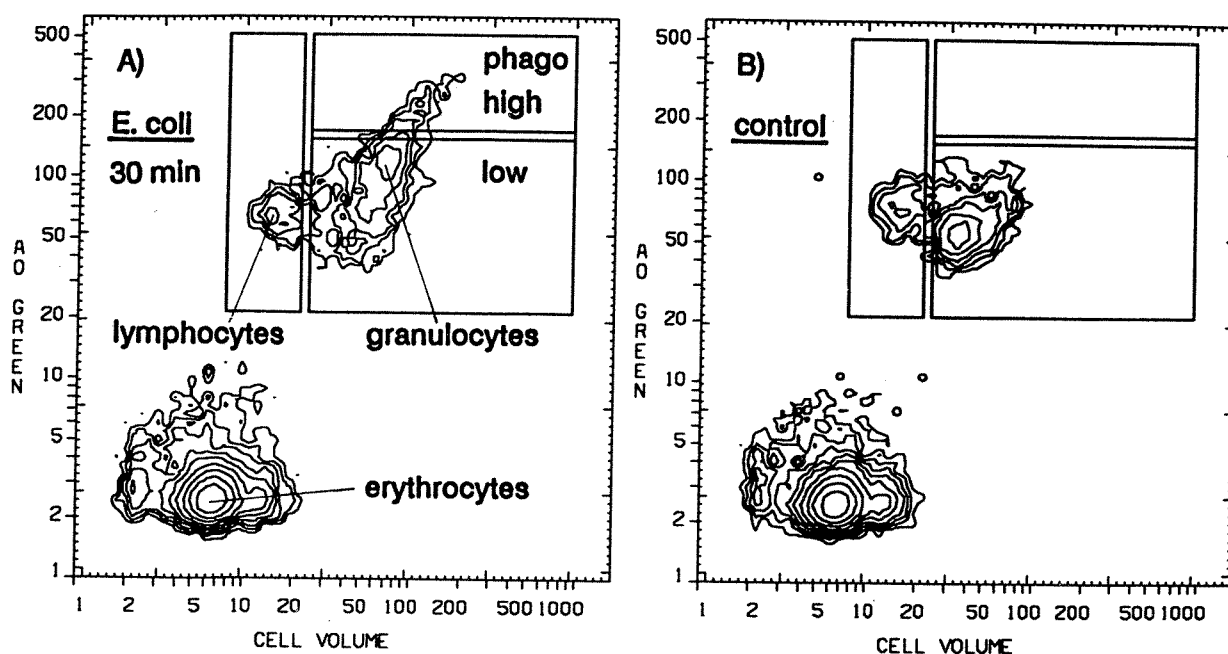
incubation of the bacteria in HEPES-buffered RPMI-1640 (Gibco BRL, Eggenstein, FRG) at 37° C with 95% air and 5% CO<sub>2</sub>. The bacteria (5 ml) were washed twice with 10 ml HBS (0.15 mol/L NaCl, 5 mmol/L HEPES, pH 7.35; SERVA Feinbiochemica, Heidelberg, FRG) and resuspended in HBS at a concentration of  $7 \times 10^9$  bacteria/ml.

**Phagocytosis.** The leukocyte suspension in autologous heparinized plasma (50  $\mu$ l) was incubated with 5  $\mu$ l of *E. coli* suspension ( $7 \times 10^9$  bacteria/ml HBS) at 37° C. A control sample was incubated with 5  $\mu$ l HBS in parallel. Aliquots (10  $\mu$ l) were taken after 0, 30, and 90 minutes, diluted with 1 ml cold HBS and stored on ice for a maximum of 2 hours.

**Fluorescent staining.** Phagocytosis and degradation of bacteria by neutrophils were measured after staining with AO (Sigma). The diluted cell suspension (250  $\mu$ l) was incubated for 15 minutes at 4° C with 5  $\mu$ l of a dye cocktail containing 0.96 mmol/L of AO and 3 mmol/L PI (Sigma) in DMF (E. Merck, Darmstadt, FRG). AO stains the DNA of leukocytes and viable bacteria green and neutrophil granule content, single-stranded nucleic acids, and degraded nucleic acids of dead bacteria red.<sup>13</sup> The increase of AO green fluorescence during phagocytosis of *E. coli* indicates the amount of ingested bacterial DNA. The initial decrease of neutrophil AO red fluorescence at 30 minutes is caused by degranulation and the increase of AO red fluorescence of phagocytosing neutrophils between 30 and 90 minutes is a measure of the intracellular degradation of bacteria. The DNA of dead leukocytes was counterstained by the PI of the dye cocktail.

Intracellular pH and esterase activity were determined after staining with ADB (Paesel, Frankfurt/Main, FRG). The diluted cell suspension (250  $\mu$ l) was incubated for 15 min at 20° C with 5  $\mu$ l of a dye cocktail containing 4.1 mmol/L ADB and 3 mmol/L PI in DMF. ADB is cleaved intracellularly by esterases into the fluorescent pH indicator DCH and non-fluorescent acetate. The total fluorescence of the cells is a measure of esterase activity. The ratio of cellular blue to green fluorescence indicates the intracellular pH according to a calibration curve.<sup>15</sup>

**Flow cytometry.** The electrical cell volume and two fluorescences of more than 2000 leukocytes per sample were measured simultaneously with a FLUVO-II flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG). The electrical cell volume was measured with HBS as sheath fluid after hydrodynamic focusing of the cells through the center of a cylindrical orifice of 80- $\mu$ m diameter and 80- $\mu$ m length at an electrical current of 0.15 mA and a suction of  $10^4$  Nm<sup>-2</sup>. Fluorescence was excited with an HBO-100 high-pressure mercury arc lamp (Osram, Augsburg, FRG). The fluorescence of AO was excited between 450 and 500 nm and measured between 500 and 530 nm (AO green fluorescence) and above 550 nm (AO and PI red fluorescence). DCH fluorescence was excited between 340 and 400 nm and the emission was collected between 420 and 440 nm (DCH blue fluorescence) and above 500 nm (DCH green fluorescence and PI red fluorescence). The flow cytometer was calibrated with porous, NH<sub>2</sub>-group-bearing particles (Paesel) of 5  $\mu$ m diameter stained with DCH (Paesel) or fluorescein isothiocyanate (Sigma). The



**Fig. 1.** Increase of neutrophil AO green fluorescence and cell volume as indicators for phagocytosis of bacteria. Buffy coat leukocytes were incubated for 30 minutes, with (A) or without (B) viable *E. coli* at 37° C, followed by staining with AO. Increase of AO green fluorescence is a measure of ingested bacterial DNA. Increase of neutrophil cell volume is a measure of cellular activation during phagocytosis. Dead cells were excluded from the analysis by simultaneous counterstaining with PI.

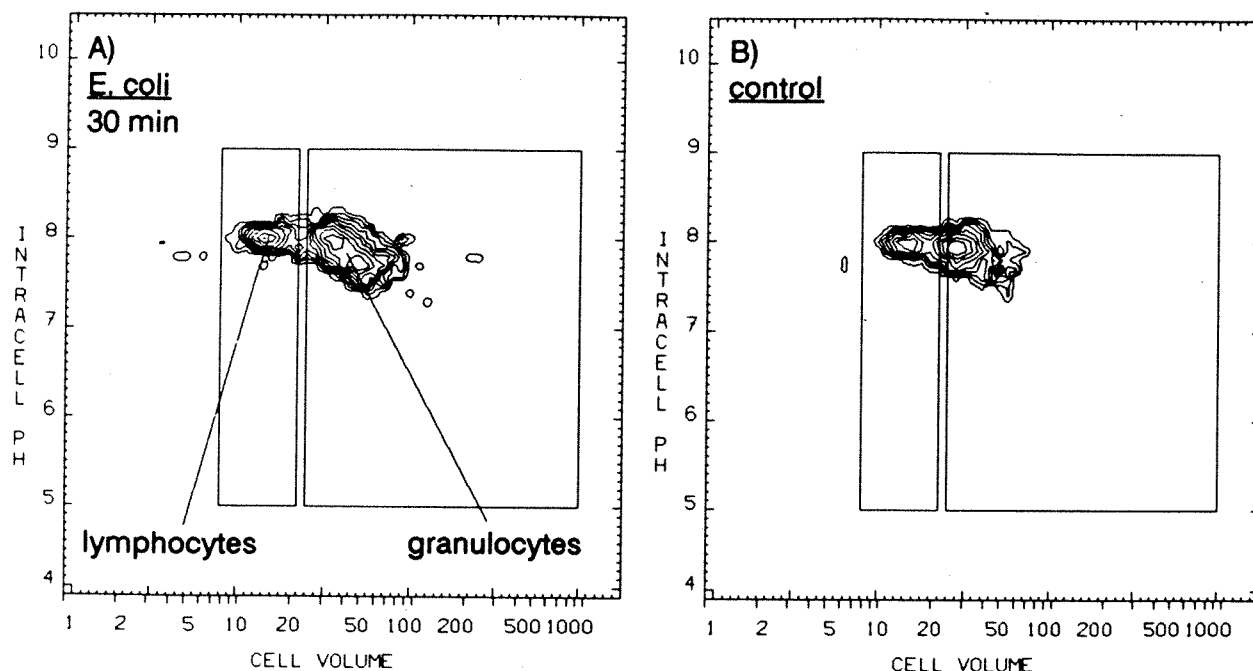
three signals of each cell were amplified logarithmically, digitized at maximum pulse height by 4096-step analog to digital converters and stored as list-mode data on magnetic tape.

**Flow cytometric data evaluation.** A graphic representation and a database of 32 parameters per sample were calculated from the flow cytometric list-mode data with the DIAGNOS1 program system<sup>16</sup> (HEKA) on an IBM-AT compatible personal computer. Vital cells and dead cells were analyzed separately by gating the list mode data in a first step for vital cells by their AO green fluorescence or by their DCH blue fluorescence, and in a second step for dead cells by their high PI red fluorescence.<sup>13</sup> The mean values of cell volume (1), AO green (2) and red (3) fluorescence, intracellular pH (4), and esterase activity (5) of viable neutrophils, and the percentage of dead cells (6) were calculated for each of the five incubations (control at 0, 30, and 90 minutes, and *E. coli* at 30 and 90 minutes) from fixed windows in the two parameter histograms obtained after gating on vital or dead cells by the program system giving 30 parameters of neutrophil function. In addition, the percentage of neutrophils was either calculated on the basis of higher AO red fluorescence of neutrophils as compared to monocytes and lymphocytes or on the basis of higher cell volume of neutrophils and monocytes than lymphocytes and the 32 parameters were transferred to the database. Monocyte activity was not evaluated separately because of considerable overlap of the monocyte cluster with lymphocytes and neutrophils.<sup>13</sup>

**Clinical data evaluation.** Age, sex, diagnosis, trauma score according to Oestern et al.,<sup>14</sup> length of ICU stay, and health status at hospital discharge were recorded for each

patient. The patient's current pulmonary and cardiovascular organ function was classified daily into one of four categories: posttraumatic organ failure, septic organ failure, transition state, or normal function after recovery. A patient was classified into the category organ failure when at least one of the following parameters was above or below the following thresholds: a ratio of the arterial oxygen partial pressure to the fraction of inspired oxygen ( $\text{PaO}_2/\text{FiO}_2$ )  $\leq 280$  according to Pepe et al.,<sup>17</sup> a  $\text{FiO}_2 > 0.5$ , mechanical ventilation with a positive endexpiratory pressure  $> 5$  cm  $\text{H}_2\text{O}$ , a mean arterial pressure below 70 mm Hg, or the requirement of positive inotropic support (dopamine hydrochloride  $\geq 5$   $\mu\text{g kg}^{-1} \text{min}^{-1}$ ) according to Skau et al.<sup>18</sup> If pulmonary and/or cardiovascular organ failure occurred within 6 days after the trauma and in the absence of infection, it was classified as posttraumatic organ failure. Pulmonary and/or cardiovascular organ failure during sepsis as defined by Montgomery et al.,<sup>19</sup> with the additional demonstration of an infectious focus or a positive blood culture, was classified as septic organ failure. The transition state was assumed when the patients did not fulfill the criteria for organ failure, but either required oxygen supply, ventilation, inotropic support, or intravenous volume therapy. A patient was classified as a recovered ICU patient when no pulmonary or cardiovascular support was required.

**Statistics.** Data are presented as the mean and the standard error of the mean. A statistical analysis of the flow cytometric data was calculated with the SPSS/PC + V2.0 program system for personal computers (SPSS, Chicago, Ill.). Significance of differences between the four clinical groups of patients was tested by the Kruskal-Wallis one-way analysis of



**Fig. 2.** Decrease of intracellular pH and increase of cell volume of neutrophils during phagocytosis of bacteria. Buffy coat leukocytes were incubated for 30 minutes, with (A) or without (B) viable *E. coli* at 37° C, followed by staining with ADB. ADB is cleaved intracellularly by esterases to the fluorescent pH indicator DCH and acetate. Intracellular pH was calculated from the ratio of cellular blue to green fluorescence, according to a calibration curve in the computer program. Dead cells were excluded from the analysis by simultaneous counterstaining with PI.

**Table I.** Functional parameters of neutrophils in septic and posttraumatic ICU patients

	Neutrophils			All leukocytes
	AO green fluorescence (arbitrary units)	Intracellular pH	Cell volume ( $\mu\text{m}^3$ )	Percentage of neutrophils
Posttraumatic organ failure (n = 22)	1.099 $\pm$ 0.067	7.46 $\pm$ 0.06	373.5 $\pm$ 23.8*	76.8 $\pm$ 2.4*
Septic organ failure (n = 28)	0.935 $\pm$ 0.049	7.64 $\pm$ 0.05*	381.3 $\pm$ 13.8*	73.6 $\pm$ 1.9*
Transition state (n = 119)	0.981 $\pm$ 0.026*	7.56 $\pm$ 0.02*	350.4 $\pm$ 4.8*	69.9 $\pm$ 1.0*
ICU patients after recovery (n = 27)	0.854 $\pm$ 0.037	7.37 $\pm$ 0.06	305.9 $\pm$ 5.7	50.7 $\pm$ 2.3
p Value	0.031†	0.001†	< 0.001†	< 0.001†
All ICU patients (n = 201)	0.970 $\pm$ 0.020	7.54 $\pm$ 0.02	351.1 $\pm$ 4.7	68.5 $\pm$ 1.0
Healthy normals (n = 24)	1.082 $\pm$ 0.067	7.55 $\pm$ 0.03	358.6 $\pm$ 9.9	34.6 $\pm$ 3.2‡

Values are expressed as mean  $\pm$  SEM.

\* $p < 0.05$ , significant differences of values of posttraumatic, septic, or transition state patients when compared to values of ICU patients after recovery (Kruskal-Wallis one-way analysis of variance).

† $p < 0.05$ , significant differences between values of the four groups of patients (Kruskal-Wallis one-way analysis of variance).

‡ $p < 0.05$ , significant differences of values between all ICU patients and healthy normal patients (Kruskal-Wallis one-way analysis of variance).

**Table II.** Effect of *E. coli* on neutrophils of septic and posttraumatic ICU patients and healthy individuals

	Increase of AO green fluorescence (% of control)		Change of intracellular pH ( $\Delta$ pH)		Increase of cell volume (% of control)	
	30 min	90 min	30 min	90 min	30 min	90 min
Posttraumatic organ failure (n = 22)	125.4 $\pm$ 4.9	135.1 $\pm$ 6.0	-0.12 $\pm$ 0.04	-0.02 $\pm$ 0.03	158.9 $\pm$ 7.8*	140.3 $\pm$ 7.3
Septic organ failure (n = 28)	122.2 $\pm$ 5.0	130.4 $\pm$ 5.1	-0.04 $\pm$ 0.02	-0.07 $\pm$ 0.04	140.5 $\pm$ 3.5	129.0 $\pm$ 4.0
Transition state (n = 119)	122.1 $\pm$ 2.1	132.9 $\pm$ 2.1	-0.06 $\pm$ 0.01	-0.04 $\pm$ 0.01	140.6 $\pm$ 2.0	127.3 $\pm$ 1.9
ICU patients after recovery (n = 27)	129.5 $\pm$ 7.8	141.2 $\pm$ 5.9	-0.04 $\pm$ 0.03	-0.04 $\pm$ 0.02	133.3 $\pm$ 3.8	122.5 $\pm$ 2.6
p Value	0.894	0.411	0.220	0.836	0.026†	0.411
All ICU patients (n = 201)	123.5 $\pm$ 1.9	133.9 $\pm$ 1.8	-0.06 $\pm$ 0.01	-0.04 $\pm$ 0.01	141.5 $\pm$ 1.7	128.3 $\pm$ 1.6
Healthy normals (n = 24)	134.2 $\pm$ 5.8	156.9 $\pm$ 6.6‡	-0.07 $\pm$ 0.02	-0.04 $\pm$ 0.02	150.7 $\pm$ 3.2‡	160.9 $\pm$ 5.0‡

Values are expressed as mean  $\pm$  SEM.

\* $p < 0.05$ , significant differences of values of posttraumatic, septic, or transition state patients when compared to values of ICU patients after recovery (Kruskal-Wallis one-way analysis of variance).

† $p < 0.05$ , significant differences between values of the four groups of patients (Kruskal-Wallis one-way analysis of variance).

‡ $p < 0.05$ , significant differences of values between all ICU patients and healthy normals (Kruskal-Wallis one-way analysis of variance).

variance ( $p < 0.05$ ).<sup>20</sup> In a *first classification* scheme, the sensitivity and specificity of the 32-flow cytometric parameter pattern for correct recognition of the *present* clinical state of the patient was analyzed by a discriminant analysis (stepwise reduction of Wilk's lambda).<sup>21</sup> Each cell sample was classified by three discriminant scores using Bayes' rule. The *predictive* sensitivity and specificity of the cell biochemical parameter pattern for the patient's clinical state on the third day after the flow cytometric measurement was tested with a *second classification* scheme, derived from the combination of the flow cytometric data of each sample, with the clinical rating of the patient on the third day after measurement of the sample.

## RESULTS

**Incidence of pulmonary or cardiovascular organ failure.** Posttraumatic (n = 13) or septic (n = 19) pulmonary and/or cardiovascular organ failure occurred in 59.6% of the 47 patients. Sepsis developed in four patients after trauma. A total of 28 of the 201 samples were obtained during episodes of sepsis and 22 samples were obtained during posttraumatic organ failure.

**Neutrophil function of normal individuals.** The phagocytic response of neutrophils from healthy individuals was characterized by three parameters: increase of cell volume (Fig. 1), increase of AO green fluorescence (Fig. 1), and decrease of the intracellular pH value (Fig. 2). The neutrophil cell volume increased from a mean value of 358.6  $\mu\text{m}^3$  before incubation (Table I) to 150.7% of this value after 30 minutes of incubation with *E. coli* K12 (Table II). The AO green fluorescence

increased from 1.082 arbitrary green fluorescence units in control cells (Table I) to 134.2% of this value after 30 minutes of phagocytosis (Table II). During incubation with *E. coli* for 90 minutes, the cell volume increased to 160.9% and AO green fluorescence to 156.9% of controls. The intracellular pH of normal neutrophils was 7.55 before incubation (Table I) and decreased by 0.07 and 0.04 pH units after 30 and 90 minutes, respectively, of incubation with bacteria (Table II).

**Neutrophil function of ICU patients.** Significant differences occurred when neutrophils from ICU patients were incubated with *E. coli* bacteria. The ingestion of bacteria and the increase of cell volume were significantly lower in neutrophils from ICU patients than in normal neutrophils. The mean cell volume of ICU patient neutrophils increased to only 141.5% of controls after 30 minutes of incubation with *E. coli* and decreased again to 128.3% after 90 minutes (Table II). The mean AO green fluorescence reached only 123.5% and 133.9% of controls after 30 minutes and 90 minutes, respectively. The intracellular pH during phagocytosis of *E. coli* decreased by 0.06 pH units and 0.04 pH units after 30 minutes and 90 minutes, respectively.

Blood samples from all ICU patients together showed a significantly higher percentage of neutrophils, but the cell volume, AO green fluorescence, or intracellular pH of the neutrophils before incubation with *E. coli* (Table I) was not significantly different from neutrophils of healthy individuals.

**Table IIIA.** Flow cytometric discriminants of current septic and posttraumatic organ failure

Rank	Posttraumatic organ failure			Septic organ failure		
	Parameter	Incubation	Stand. coeff.	Parameter	Incubation	Stand. coeff.
1	Increase of AO green fluorescence.	<i>E. coli</i> , 90 min	1.502	Cell volume	Control, 90 min	0.953
2	Percentage of neutrophils	Control, 0 min	1.225	Percentage of neutrophils	Control, 0 min	0.797
3	Increase of AO green fluorescence.	<i>E. coli</i> , 30 min	0.994	Cell volume	Control, 30 min	0.603
4	Cell volume	Control, 0 min	0.513	Increase of AO green fluorescence.	<i>E. coli</i> , 90 min	0.549
5	Cell volume	Control, 90 min	0.446	Increase of cell volume	<i>E. coli</i> , 90 min	0.337

**Table IIIB.** Flow cytometric discriminants of imminent septic and posttraumatic organ failure

Rank	Posttraumatic organ failure			Septic organ failure		
	Parameter	Incubation	Stand. coeff.	Parameter	Incubation	Stand. coeff.
1	Percentage of neutrophils	Control, 0 min	1.239	Increase of AO green fluorescence	<i>E. coli</i> , 30 min	1.819
2	Cell volume	Control, 90 min	1.124	Cell volume	Control, 90 min	1.436
3	Cell volume	Control, 30 min	0.867	Decrease of intracellular pH	<i>E. coli</i> , 90 min	1.307
4	Increase of AO green fluorescence	<i>E. coli</i> , 30 min	0.720	Increase of cell death	<i>E. coli</i> , 90 min	1.175
5	Intracellular pH	Control, 0 min	0.603	AO green fluorescence	Control, 30 min	0.799

List of five flow cytometric parameters with highest standardized discriminant coefficients in a multifactorial analysis. All 32 flow cytometric parameters were considered for automated discrimination of either current or imminent posttraumatic or septic organ failure. Samples from recovered ICU patients served as control group.

**Longitudinal study of neutrophils in individual patients.** Typical posttraumatic time courses of the phagocytic response of neutrophils in individual patients showed high increases of neutrophil cell volume during phagocytosis in the early posttraumatic phase, followed by rapid declines of cell volume increase already several days before the onset of septic periods (Fig. 3, A). The intracellular pH of control neutrophils and neutrophils incubated with *E. coli* also exhibited a typical pattern with acidic intracellular pH values after trauma and alkaline pH during septic episodes (Fig. 3, B).

**Significance of disease-related cell biochemical parameter patterns.** The grouping of the blood samples according to the daily classification of the patients' pulmonary and vascular organ function revealed significant disease-related parameter patterns for resting (Table I) and phagocytosing (Table II) neutrophils. Blood samples obtained from septic or posttraumatic patients during pulmonary or cardiovascular dysfunction contained neutrophils with a larger mean cell volume and a higher percentage of neutrophils (Table I). An alkaline intracellular pH was associated with sepsis and a high accumulation of AO green fluorescence by neutrophils was observed in the posttraumatic state.

The increase of cell volume during incubation with *E. coli* was significantly higher during posttraumatic organ failure (Table II). The lower phagocytosis of bacteria in septic samples, measured by the increase of AO green fluorescence, and the higher decrease of the intracellular pH in posttraumatic samples were not significantly different from samples obtained from ICU patients after recovery.

**Extracellular pH and carbon dioxide tension in ICU patients.** In order to correlate intracellular pH values with respiratory acid-base disturbances, the extracellular pH and carbon dioxide tension of arterial blood were determined. The mean arterial pH value in the four groups of patients did not differ significantly, with a range from 7.38 to 7.41 pH units. The arterial carbon dioxide tension had a mean of  $41.7 \pm 1.0$  mm Hg for all patients and was significantly different between the four groups of patients, with the lowest values in recovered ICU patient samples ( $34.1 \pm 1.8$  mm Hg), the highest values in transition state samples ( $43.6 \pm 1.4$  mm Hg), and intermediate values during posttraumatic ( $39.8 \pm 2.2$  mm Hg) or septic organ failure ( $41.8 \pm 1.5$  mm Hg).

**Automated classification by flow cytometric param-**

**Table IV.** Automated classification of intensive care patients with flow cytometric parameters of neutrophil function for current clinical condition

Clinical diagnosis	n	DIAGNOSIS/SPSS-classification*			
		Posttraumatic organ failure	Septic organ failure	Transition state	Normal ICU patients
Posttraumatic organ failure	20	90.0	0.0	10.0	0.0
Septic organ failure	27	0.0	77.8	22.2	0.0
Transition state	113	7.1	14.2	75.2	3.5
ICU patients after recovery	26	0.0	0.0	11.5	88.5

Classification by three discriminant scores was derived by a stepwise minimization of Wilks' lambda from 32 flow cytometric parameters of neutrophil function.

\*Values are expressed in percentage.

**Table V.** Automated three day in advance classification of intensive care patients with flow cytometric parameters of neutrophil function for imminent clinical condition

Clinical diagnosis 3 days later	n	DIAGNOSIS/SPSS-classification*			
		Posttraumatic organ failure	Septic organ failure	Transition state	Normal ICU patients
Posttraumatic organ failure	8	100.0	0.0	0.0	0.0
Septic organ failure	12	0.0	91.7	8.3	0.0
Transition state	94	1.1	7.4	87.2	4.3
ICU patient after recovery	23	0.0	4.3	0.0	95.7

Classification by three discriminant scores was derived by a stepwise minimization of Wilks' lambda from 32 flow cytometric parameters of neutrophil function.

\*Values are expressed in percentage.

**eters.** The sensitivity and specificity of the flow cytometric parameters for the identification of the patient's current disease status were analyzed by a discriminant analysis. This stepwise procedure combined the various flow cytometric parameters in increasing number with the aim to improve the discrimination of the four clinical groups of samples (i.e., to minimize the variability of the three discriminant scores within each clinical group, as compared to the variability of the three scores for all patients). The increase of AO green fluorescence during phagocytosis, the cell volume of resting neutrophils, and the percentage of neutrophils were the most significant discriminators when either posttraumatic or septic samples were compared with samples obtained from recovered ICU patients (Table IIIA and IIIB).

Ultimately, the combination of 26 of the 32 determined flow cytometric parameters to three discriminant scores was optimal, with the correct identification of 90.0% of the posttraumatic samples and 77.8% of the septic samples (Table IV). The remainder of the post-

traumatic and septic samples were misclassified as transition state. No sample from a recovered ICU patient was misclassified as a posttraumatic or septic sample.

The predictive sensitivity of the flow cytometric patterns of neutrophil function was analyzed by combining the flow cytometric data of each sample with the clinical rating of the same patient on the third day after measurement of the sample. The discriminant analysis of these data led to a second classification algorithm (Tables IIIA and IIIB), which correctly predicted pulmonary or cardiovascular organ failure in posttraumatic or septic patients by 100.0% or 91.7%, respectively (Table V).

## DISCUSSION

Flow cytometric parameters of neutrophil function, such as DNA content, intracellular pH, and cell volume during phagocytosis and degradation of *E. coli*, showed significant differences between samples from posttraumatic or septic ICU patients and a healthy con-



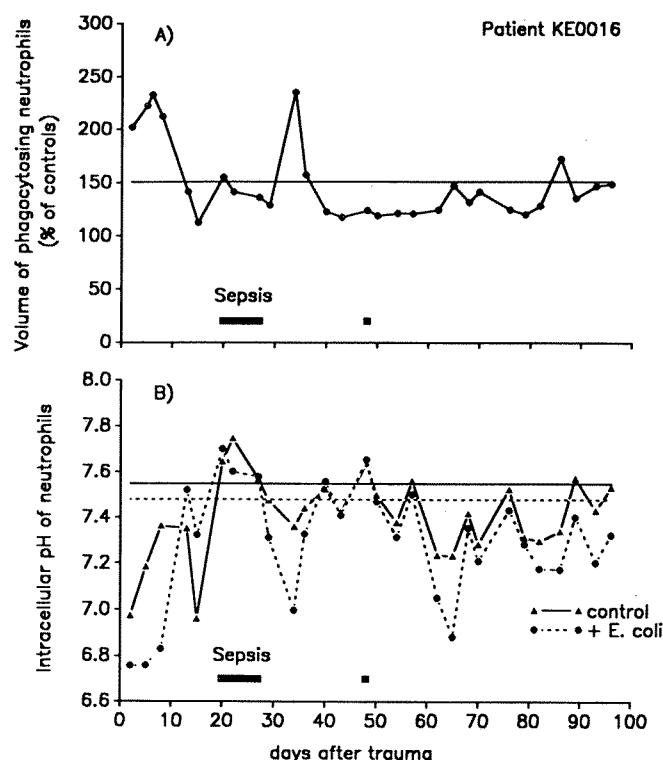
trol group (Tables I and II). The combination of several of these parameters to a multifunctional parameter pattern allowed the discrimination of patients with post-traumatic pulmonary or cardiovascular organ failure from patients with septic organ failure and patients in transition state or with stable organ function (Tables IIIA, IIIB, and IV). Furthermore, the course of the disease was predicted 3 days in advance (Table V).

The ingestion and intracellular degradation of bacteria was measured by staining vital cells with the metachromatic dye AO. AO fluoresces green when intercalating into double-stranded nucleic acids and fluoresces red when complexing with single-stranded or denatured nucleic acids.<sup>22</sup> The ingestion of bacterial DNA by neutrophils was lower in blood samples from ICU patients than in blood samples from a healthy reference group (Table II). The four groups of ICU patients did not differ significantly in the increase of AO green fluorescence, indicating no trauma- or sepsis-associated alterations of the capability of neutrophils to ingest bacteria, a property of neutrophils that is already developed at the metamyelocyte state.<sup>23</sup> The incubation of unseparated leukocytes in autologous plasma with a twenty-fold excess of *E. coli* in our assay neither limited phagocytosis by low opsonization nor by low numbers of bacteria.<sup>13</sup>

The vital staining of resting neutrophils with AO also allowed the quantitative analysis of their nuclear structure, as changes of chromatin condensation affect the intercalative binding of AO to DNA.<sup>24</sup> The AO green fluorescence of neutrophils was 29% higher in samples from patients with posttraumatic pulmonary or cardiovascular organ failure than in samples from patients with stabilized organ function (Table I), suggesting significant disease-related alterations of the nuclear structure of neutrophils.

The intracellular pH of resting and phagocytosing neutrophils was analyzed as a parameter of the cellular metabolic activity.<sup>25</sup> The intracellular pH is regulated by an electroneutral  $\text{Na}^+/\text{H}^+$  exchange in neutrophils and is tightly related to cell activation. An alkaline intracellular pH increases receptor-coupled reactions leading to the expression of NADPH oxidase, which releases superoxide anion in stimulated neutrophils.<sup>26,27</sup> Upon cellular activation, the electron-translocating NADPH oxidase induces an initial cytoplasmic acidification through the generation of large amounts of protons. The protons are transferred to the granules and to the exterior of the cells by  $\text{Zn}^{2+}$ - or  $\text{Cd}^{2+}$ -sensitive proton-conducting channels.<sup>28</sup> In addition, the  $\text{Na}^+/\text{H}^+$  exchange is activated. This leads to a sustained intracellular alkalization.<sup>29</sup> It also induces chemotaxis<sup>30</sup> and an increase of the cell volume of neutrophils.<sup>31</sup>

The intracellular pH values of resting neutrophils from the four groups of patients were significantly dif-



**Fig. 3.** Longitudinal study of neutrophil function in a posttraumatic patient. **A**, increase of neutrophil cell volume during phagocytosis of *E. coli*. Buffy coat leukocytes were incubated with *E. coli* for 30 minutes at 37° C, stained with AO, and analyzed as described in Fig. 1. Cell volume of *E. coli*-incubated neutrophils is given as percentage of cell volume of neutrophils incubated without bacteria. **B**, intracellular pH of neutrophils. Buffy coat leukocytes were incubated with (●—●) or without (▲—▲) *E. coli* for 30 minutes at 37° C, stained with ADB, and analyzed as described in Fig. 2. Straight lines indicate the mean values for a reference group of healthy individuals (n = 14).

ferent. Alkaline pH values were observed during septic pulmonary or cardiovascular organ failure and acidic pH values during stable organ function after patients' recovery (Table I). No differences of the extracellular blood arterial pH were observed in the four groups of patients. The alkaline intracellular pH and the large cell volume of neutrophils observed during septic organ failure reflected metabolic activation of the neutrophils because they were accompanied by a normal arterial carbon dioxide tension. Despite the different intracellular pH values of resting neutrophils, the slight acidification of the intracellular pH after phagocytosis was similar in all patient groups (Table II), indicating intact regulation of the acid-balance during the respiratory burst.

The increase of neutrophil cell volume during phagocytosis of *E. coli* was a more sensitive indicator of the activation of the phagocytic process after trauma than the ingestion of *E. coli* as measured by increase of AO green fluorescence (Table II). Furthermore, in all groups of patients—but not in healthy normals—the

increased cell volume of phagocytosing neutrophils declined after 90 minutes of incubation with *E. coli*, despite continuous ingestion of *E. coli* as indicated by increasing AO green fluorescence (Table II). This shows that the change of cell volume during phagocytosis was not only caused by the volume of the ingested bacteria, but in addition indicated active cellular processes, like the reversible cell swelling induced by activated complement,<sup>32,33</sup> phorbol esters, or chemotactic peptides.<sup>31</sup>

The resting cell volume of neutrophils was also a sensitive indicator for both posttraumatic and septic organ failure (Tables I, IIIA, and IIIB). This confirms centrifugal elutriation studies that showed that the resting cell volume of human peripheral blood neutrophils is not correlated with cell age, but associated with increased NADPH oxidase activity,<sup>34</sup> possibly as a consequence of earlier activation of the cell.<sup>35</sup>

The multifunctional flow cytometric analysis of phagocytosing and control-incubated neutrophils revealed two patterns of abnormal neutrophil function in ICU patients (Tables IIIA and IIIB). The posttraumatic activation of neutrophils was associated with an increased functional response to phagocytosis of *E. coli*, as measured by increased cell swelling and increased acidification, whereas neutrophils from septic patients exhibited pre-existing high cell volume and alkaline pH (Table I) and showed low responses when incubated with *E. coli* (Table II). The increase of neutrophil counts and the increase of their resting cell volume and their AO green fluorescence were indicators of neutrophil activation during the transition state of patients.

The activated functional state of neutrophils during posttraumatic pulmonary or cardiovascular organ failure may result from the recruitment of neutrophils from the marginated blood vessel cell pool or from the bone marrow cell pool. Neutrophils derived from the bone marrow have been reported to be functionally different from peripheral blood neutrophils in some studies,<sup>36,37</sup> but not in others.<sup>38</sup> Alternatively, the hyperergic phagocytosis of neutrophils trauma may result from activation of normal peripheral blood neutrophils (e.g., by complement cleavage products). During septic organ failure, bacterial products like endotoxin,<sup>35,39,40</sup> or chemotactic bacterial peptides,<sup>41</sup> or immune-mediators released from monocytes<sup>42</sup> and macrophages<sup>43</sup> like interleukin-1 or tumor necrosis factor alpha<sup>44-46</sup> are liberated and may be responsible for the alkaline pH and large cell volume of neutrophils and their lower responses to incubation with *E. coli*.

The results of this study suggest that systemic alterations of neutrophil function—rather than only an alteration of the local milieu—are associated with the imminence of posttraumatic or septic multiple organ failure. The longitudinal analysis of such alterations of

neutrophil function with sensitive multiparametric cell-biochemical techniques is important not only for the early recognition of imminent sepsis, but also for the understanding of the mechanisms of the alterations of neutrophil function during posttraumatic and septic organ failure in ICU patients.

## REFERENCES

1. Malech HL, Gallin JJ. Current concepts: Immunology. Neutrophils in human diseases. *N Engl J Med* 1987;317:687-94.
2. Cohen AB, MacArthur C, Idell S, et al. A peptide from alveolar macrophages that releases neutrophil enzymes into the lungs in patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1988;137:1151-8.
3. Zimmerman GA, Renzetti AD, Hill HR. Functional and metabolic activity of granulocytes from patients with adult respiratory distress syndrome. Evidence for activated neutrophils in the pulmonary circulation. *Am Rev Respir Dis* 1983;127:290-300.
4. Bass DA, Olbrantz P, Szejda P, Seeds MC, McCall CE. Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. *J Immunol* 1986;136:860-6.
5. Ozaki Y, Ohashi T, Niwa Y. Evaluation of neutrophil acetyltransferase activity in patients with inflammatory disorders. *Life Sciences* 1986;39:2343-50.
6. Barbour AG, Craig CD, Solberg CO, Hill HR. Chemiluminescence by polymorphonuclear leukocytes from patients with active bacterial infection. *J Infect Dis* 1980;141:14-26.
7. Venge P, Strömberg A, Braconier JH, Roxin LE, Olsson I. Neutrophil and eosinophil granulocytes in bacterial infection: sequential studies of cellular and serum levels of granule proteins. *Br J Haematol* 1978;38:475-83.
8. Marino JA, Gerding RL, Fratianni RB, Spagnuolo PJ. Neutrophil adhesive dysfunction in thermal injury: the role of fibronectin. *J Infect Dis* 1988;157:674-81.
9. Davis CF, Moore FD, Rodrick ML, Fearon DT, Mannick JA. Neutrophil activation after burn injury: contributions of the classic complement pathway and endotoxin. *Surgery* 1987;102:477-84.
10. Dwenger A, Schweitzer G, Regel G. Bronchoalveolar lavage fluid and plasma proteins, chemiluminescence response and protein contents of polymorphonuclear leukocytes from blood and lavage fluid in traumatized patients. *J Clin Chem Clin Biochem* 1986;24:73-88.
11. Green DR, Faist E. Trauma and the immune response. *Immunology Today* 1988;9:253-5.
12. Shapiro MF, Greenfield S. The complete blood count and leukocyte differential count. An approach to their rational application. *Ann Intern Med* 1987;106:65-74.
13. Rothe G, Valet G. Phagocytosis, intracellular pH, and cell volume in the multifunctional analysis of granulocytes by flow cytometry. *Cytometry* 1988;9:316-24.
14. Oestern HJ, Sturm JA, Tscherne H. Die Klassifizierung der Verletzungsschwere. *Hefte Unfallheilk* 1983;156:171-6.
15. Valet G, Raffael A, Moroder L, Wuensch E, Ruhenstroth-Bauer G. Fast intracellular pH determination in single cells by flow cytometry. *Naturwissenschaften* 1981;68:265-6.
16. Valet G, Warnecke HH, Kahle H. Automated diagnosis of malignant and other abnormal cells by flow-cytometry using the DIAGNOS1 program system. In: Burger G, Ploem JS, Goerttler K, eds. *Clinical cytometry and histometry*. London: Academic Press, 1987:58-65.
17. Pepe PE, Potkin RT, Reus DH, Hudson LD, Carrico CJ. Clinical

- predictors of the adult respiratory distress syndrome. *Am J Surg* 1987;144:124-30.
18. Skau T, Nyström PO, Carlsson C. Severity of illness in intra-abdominal infection: a comparison of two indexes. *Arch Surg* 1985;120:152-8.
19. Montgomery AB, Stager MA, Carrico CJ, Hudson L. Causes of mortality in patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1985;132:485-9.
20. Norusis MJ. SPSS/PC+ V2.0 base manual. Chicago: SPSS Inc., 1988.
21. Norusis MJ. SPSS/PC+ V2.0 advanced statistics. Chicago: SPSS Inc., 1988.
22. Darzynkiewicz Z, Traganos F, Kapuscinski J, Melamed MR. Denaturation and condensation of DNA in situ induced by acridine orange in relation to chromatin changes during growth and differentiation of friend erythroleukemia cells. *Cytometry* 1985;6:195-207.
23. Glasser L, Fiederlein RL. Functional differentiation of normal human neutrophils. *Blood* 1987;69:937-44.
24. Traganos F, Darzynkiewicz Z, Sharpless T, Melamed MR. Cytofluorometric studies on conformation of nucleic acids in situ. I. Restriction of acridine orange binding by chromatin proteins. *J Histochem Cytochem* 1976;24:40-8.
25. Frelin C, Vigne P, Ladoux A, Lazdunski M. The regulation of the intracellular pH in cells from vertebrates. *Eur J Biochem* 1988;174:3-14.
26. Wright J, Maridonneau-Parini I, Cragoe EJ, Schwartz JH, Tauber AI. The role of the  $\text{Na}^+/\text{H}^+$  antiporter in human neutrophil NADPH-oxidase activation. *J Leukocyte Biol* 1988;43:183-6.
27. Simchowitz L. Intracellular pH modulates the generation of superoxide radicals by human neutrophils. *J Clin Invest* 1985;76:1079-89.
28. Henderson LM, Chappell JB, Jones OTG. Internal pH changes associated with the activity of NADPH oxidase of human neutrophils: further evidence for the presence of an  $\text{H}^+$  conducting channel. *Biochem J* 1988;251:563-7.
29. Weisman SJ, Punzo A, Ford C, Sha'afi RI. Intracellular pH during neutrophil activation:  $\text{Na}^+/\text{H}^+$  antiport. *J Leukocyte Biol* 1987;41:25-32.
30. Simchowitz L, Cragoe EJ. Regulation of human neutrophil chemotaxis by intracellular pH. *J Biol Chem* 1986;261:6492-500.
31. Grinstein S, Furuya W, Cragoe EJ. Volume changes in activated human neutrophils: the role of  $\text{Na}^+/\text{H}^+$  exchange. *J Cell Physiol* 1986;128:33-40.
32. O'Flaherty JTO, Kreutzer DL, Ward PA. Chemotactic factor influences on the aggregation, swelling, and foreign surface adhesiveness of human leukocytes. *Am J Pathol* 1978;90:537-50.
33. Morgan BP. Non-lethal complement attack on human neutrophils: transient cell swelling and metabolic depletion. *Immunology* 1988;63:71-7.
34. Berkow RL, Dodson RW. Volume dependent polymorphonuclear leukocytes fractions isolated by counterflow centrifugal elutriation: PMN volume does not correlate with PMN age. *J Leukocyte Biol* 1987;41:518-26.
35. Guthrie LA, McPhail L, Henson PM, Johnston RB. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide: evidence for increased activity of the superoxide-producing enzyme. *J Exp Med* 1984;160:1656-71.
36. Steele RW, Steele CR, Pilkington NS, Charlton RK. Functional capacity of marginated and bone marrow reserve granulocytes. *Infect Immun* 1987;55:2359-63.
37. Hetherington SV, Quie PG. Human polymorphonuclear leukocytes of the bone marrow, circulation, and marginated pool: Function and granule protein content. *Am J Hematol* 1985;20:235-46.
38. Berkow RL, Dodson RW. Functional analysis of the marginating pool of human polymorphonuclear leukocytes. *Am J Hematol* 1987;24:47-54.
39. Wright GG, Read PW, Mandell GL. Lipopolysaccharide releases a priming substance from platelets that augments the oxidative response of polymorphonuclear neutrophils to chemotactic peptide. *J Infect Dis* 1988;157:690-6.
40. Moore FD, Moss NA, Revhaug A, Wilmore D, Mannick JA, Rodrick ML. A single dose of endotoxin activates neutrophils without activating complement. *Surgery* 1987;102:200-5.
41. Hughes V, Humphreys JM, Edwards SW. Protein synthesis is activated in primed neutrophils: a possible role in inflammation. *Biosci Report* 1987;7:881-90.
42. Koivuranta-Vaara P, Banda D, Goldstein IM. Bacterial-lipopolysaccharide-induced release of lactoferrin from human polymorphonuclear leukocytes: role of monocyte-derived tumor necrosis factor  $\alpha$ . *Infect Immun* 1987;55:2956-61.
43. Movat HZ, Cybulski MI, Colditz IG, Chan MKW, Dinarello CA. Acute inflammation in gram-negative infection: endotoxin, interleukin 1, tumor necrosis factor, and neutrophils. *Federation Proc* 1987;46:97-104.
44. Sullivan GW, Carper HT, Novick WJ, Mandell GL. Inhibition of the inflammatory action of interleukin-1 and tumor necrosis factor (alpha) on neutrophil function by pentoxifylline. *Infect Immunol* 1988;56:1722-9.
45. Blanchard DK, Djeu JY, Klein TW, Friedman H, Stewart WE. Protective effects of tumor necrosis factor in experimental *Legionella pneumophila* infections of mice via activation of PMN function. *J Leukocyte Biol* 1988;43:429-35.
46. Ferrante A, Nandoskar M, Walz A, Goh DHB, Kowanko IC. Effect of tumour necrosis factor alpha and interleukin-1 alpha and beta on human neutrophil migration, respiratory burst, and degranulation. *Int Arch Allergy Appl Immun* 1988;86:82-91.