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High Light Scatter by Neutrophils in the Bayer-Technicon H*2 Analyzer: A Screening Test of Morphologically Defective Responsiveness to in vitro Chemotactic Stimulation

By *U. Lippi* [1], *P. Bellavite* [2], *M. Schinella* [1], *M. Nicoli* [1] and *G. Lippi* [2]

[1] *Laboratorio di Chimica Clinica ed Ematologia, Ospedale Civile Maggiore, Verona, Italy*
[2] *Istituto di Chimica e Microscopia Clinica, Università degli Studi, Verona, Italy*

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Summary: The Bayer-Technicon H*2 haematological analyser provides differential white blood cell count, including the assay of polymorphonuclear leukocytes by light scattering and the absorbance increase following the cytochemical reaction for myeloperoxidase. The mean value of polymorphonuclear leukocytes scatter, which reflects polymorphonuclear leukocytes volume, is printed in a separate report "for laboratory use only" as a ybar value in arbitrary units.

In certain patients neutrophils displayed an unreported correlation between polymorphonuclear leukocytes high ybar basal values ($\geq$ 37.00 arbitrary units) (determined on the H*2) and a defective response in vitro to the chemoattractant, formyl-methionyl-leucyl-phenylalanine (determined by microscopic evaluation of polymorphonuclear leukocytes shape change (polarization)). The patients showing no polymorphonuclear leukocyte response or a defective one to formyl-methionyl-leucyl-phenylalanine were all affected by "Systemic Inflammatory Response Syndrome (SIRS)". Therefore the predictive value of the positive test for SIRS is 100%. On the other hand 8.8% of SIRS patients had polymorphonuclear leukocytes $<$ 37.00 arbitrary units of ybar basal value and a "normal" response to formyl-methionyl-leucyl-phenylalanine; the predictive value of the negative test being 90%.

Since we demonstrated in vitro a dose-dependent deactivation of endotoxin or lipopolysaccharide-pretreated polymorphonuclear leukocytes, the "normal" response to formyl-methionyl-leucyl-phenylalanine of the "false negative" cases may occur because the endotoxaemia in these patients is too low to prevent it. Thus, high polymorphonuclear leukocyte scatter values on the H*2 allows the identification of a group of critically ill patients in whom activated neutrophils do not respond to further stimulation by polymorphonuclear leukocyte polarization, a shape change that is characteristic of migrating cells and essential for chemotaxis.

Introduction

In certain patients with a depressed white blood cell count, it has been shown that the polymorphonuclear leukocytes display a defective response to the chemoattractant, formyl-methionyl-leucyl-phenylalanine (1). This response can be evaluated in vitro by treating polymorphonuclear leukocytes with formyl-methionyl-leucyl-phenylalanine, then monitoring morphological changes (polarization) by assaying shape alterations by light scattering with aggregometry (2), using a spectrofluorometric test (3), or the microscopic count of polarized, bipolar cells (1, 4). Microscopic evaluation of formyl-methionyl-leucyl-phenylalanine-induced polymorphonuclear leukocyte bipolar shape formation on smears from whole blood is easier, more rapid to perform and the percentage of biplar cells correlates very well with polymorphonuclear leukocyte migration assay (1, 4).

To verify and confirm the correlation between leukopenia and defective bipolar shape formation, we per-

formed white blood cell counts on the Bayer-Technicon H*2 haematological analyser. In some cases of defective bipolar shape formation response to formyl-methionyl-leucyl-phenylalanine, we found an unreported reverse correlation between the magnitude of polymorphonuclear leukocyte-scattered light in the H*2 and the percentage of bipolar cells (%bipolar shape formation). Polymorphonuclear leukocyte-scattered light of neutrophils may be detected in the H*2 by visual inspection of the PEROX display, where polymorphonuclear leukocyte-scattered light values on the y-axis are plotted against the absorbance of the cytochemical myeloperoxidase reaction on the x-axis to provide a differential white blood cell count. The mean scatter values of neutrophils, which reflects cell volume, is printed in a separate report and allows quantitative estimation of polymorphonuclear leukocyte volumes.

The purpose of this paper is to establish criteria for using the H*2 to identify blood samples with increased polymorphonuclear leukocyte light scattering and to study their morphological responsiveness to formyl-methionyl-leucyl-phenylalanine. This (immuno) haematological information supplements that already provided by haematological analysers.

Materials and Methods

Venous blood samples from 20 healthy volunteers and 91 randomly selected hospitalized patients were anticoagulated with K_3-EDTA (1.5 g/l), maintained at controlled room temperature (23–25 °C) and processed at fixed times between 30 min and 4 h after venipuncture. Each sample was divided into aliquots of 0.9 ml and treated by adding 0.1 ml of formyl-methionyl-leucyl-phenylalanine (10^{-5} to 10^{-8} mol/l) solution. Formyl-methionyl-leucyl-phenylalanine (Sigma F-3506) was dissolved in dimethyl sulphoxide (Sigma D-5879) at 10^{-2} mol/l concentration, aliquoted and stored at −70 °C. Working solutions were further diluted with 0.15 mol/l NaCl immediately before use. Controls were performed with 0.1 ml of 0.15 mol/l NaCl. Five normal samples were preincubated 1 h at room temperature with lipopolysaccharide (from *Escherichia coli*, Sigma L-9023) at final concentrations ranging from 1 to 100 µg/l. After formyl-methionyl-leucyl-phenylalanine stimulation (5 to 20 min) at room temperature, both NaCl- and formyl-methionyl-leucyl-phenylalanine-treated samples were analysed using the Bayer-Technicon H*2 (Bayer-Technicon, Tarrytown, New York). Quantitative estimation of both locations of the polymorphonuclear leukocyte cluster mean, along the y-axis (scattered light) and along the x-axis (absorbance of myeloperoxidase reaction), are printed in arbitrary units as ybar and xbar values, respectively, in a table corresponding to identification (Id) number 10 of the cluster on the instrument-generated report Research Screen 2. Two further measurements, both printed on the Research Screen 2 report, are related to the myeloperoxidase activity of neutrophils: mean peroxidase index and percentage of high peroxidase cells. Mean peroxidase index shows the displacement of the mean peroxidase activity of the analysed polymorphonuclear leukocytes from the mean peroxidase activity of a "normal" polymorphonuclear leukocyte population; high peroxidase cells could reflect the percentage of cells with high myeloperoxidase activity, but it is more probably due to an alteration in myeloperoxidase distribution within the neutrophils (5). Smears from the same whole blood were fixed and stained with *May-Gruenwald Giemsa* and examined microscopically using a x50 oil objective. Fifty neutrophils from two smears were classified according to polymorphonuclear leukocyte shape, and the percentage of bipolar cells was expressed as %bipolar shape formation.

Results

The difference (Δ) between ybar values of resting and formyl-methionyl-leucyl-phenylalanine-activated neutrophils was considered as a measure of polymorphonuclear leukocyte volume changes. Bipolar shape formation was considered a morphological effect of formyl-methionyl-leucyl-phenylalanine activation.

We studied the polymorphonuclear leukocyte response in relation to formyl-methionyl-leucyl-phenylalanine concentration, time of incubation and aging of the samples. The results are summarized below.

1. The dose-response relationship showed a maximal response of ybar values at 10^{-7} mol/l formyl-methionyl-leucyl-phenylalanine, following a 15 min incubation at room temperature. At higher formyl-methionyl-leucyl-phenylalanine concentrations (10^{-6} mol/l) the dose-response of the ybar values decreased in a number of blood samples, whereas the microscopic assay of %bipolar shape formation was unchanged at formyl-methionyl-leucyl-phenylalanine concentrations ranging between 10^{-5} and 10^{-8} mol/l. Therefore a formyl-methionyl-leucyl-phenylalanine concentration of 10^{-7} mol/l was chosen for the experiments.

2. The time-response relationship at 10^{-7} mol/l formyl-methionyl-leucyl-phenylalanine showed the highest increase of ybar values after 8 min incubation at room temperature, and this increased value remained constant for at least 20 min. Since %bipolar shape formation was almost maximal at 10 min and slowly increased between 10 and 20 min (less than an additional 7%), formyl-methionyl-leucyl-phenylalanine-treated blood was smeared after a 10 min incubation.

3. Aging effects upon either ybar basal values or ybar and %bipolar shape formation, after formyl-methionyl-leucyl-phenylalanine treatment, were negligible between 30 min and 3 h after venipuncture. In fact, repeated assays performed from 30 min to 3 h after venipuncture showed satisfactory precision (CV = 6.8%) with relatively stable ybar basal values within this interval. Three and a half hours after venipuncture, basal ybar values of resting neutrophils increased dramatically and the polymorphonuclear leukocyte response to formyl-methionyl-leucyl-phenylalanine dropped.

Tab. 1 Average values ($\bar{x}$), standard deviations (SD) and ranges of resting polymorphonuclear leukocyte scatter (ybar) or cytochemical myeloperoxidase ($\bar{x}$bar) values and formyl-methionyl-leucyl-phenylalanine-induced changes (△) on ybar, xbar, mean peroxidase index (MPXI), high peroxidase cells (%HPX) and polymorphonuclear leukocyte morphology (%bipolar shape formation) (%BSF) in blood samples (n = 20) from healthy volunteers.

	ybar	△ybar	xbar	△xbar (−)	△MPXI (−)	△%HPX (+)	%BSF
$\bar{x}$	33.89	2.90	28.70	1.37	2.50	0.85	32.8
SD	0.74	0.65	0.98	0.96	2.20	0.50	5.98
Ranges	32.70−35.00	1.90−4.50	27.60−30.35	0.70−2.60	0.10−6.50	0.10−1.70	24−42

Basal values of ybar, xbar, mean peroxidase index, high peroxidase cells and formyl-methionyl-leucyl-phenylalanine-induced changes of these quantities and %bipolar shape formation on whole blood from 20 healthy volunteers are reported in table 1. The samples were assayed from 30 min to 3 h after venipuncture. Polymorphonuclear leukocyte basal scatter values of normal subjects did not exceed 35.00 arbitrary units. Formyl-methionyl-leucyl-phenylalanine induced a consistent increase of polymorphonuclear leukocyte-scattered light and high peroxidase cells, whereas both xbar values and mean peroxidase index decreased. The lowest value of %bipolar shape formation was 24. A representative case of polymorphonuclear leukocyte normal response to formyl-methionyl-leucyl-phenylalanine is shown in figure 1. The differences of ybar values in normal samples or drawn from 91 hospitalized patients are plotted against %bipolar shape formation (fig. 2). As figure 2 shows, both ybar values and %bipolar shape formation were low in 24 samples at polymorphonuclear leukocyte ybar basal values ⩾ 37.00 arbitrary units. Results of our reference method for assying polymorphonuclear leukocyte responsiveness to formyl-methionyl-leucyl-phenylalanine (bipolar shape formation) correlated poorly with the Δ ybar values. The discrepancy may be due to the fact that the H*2 also measures the size of formyl-methionyl-leucyl-phenylalanine-mildly-stimulated, large ("amorphous") neutrophils, whereas the microscopic examination of smears records bipolar cells only. Also in the case of formyl-methionyl-leucyl-phenylalanine unresponsiveness (fig. 3) the instrumental index of myeloperoxidase activity demonstrated variable myeloper-

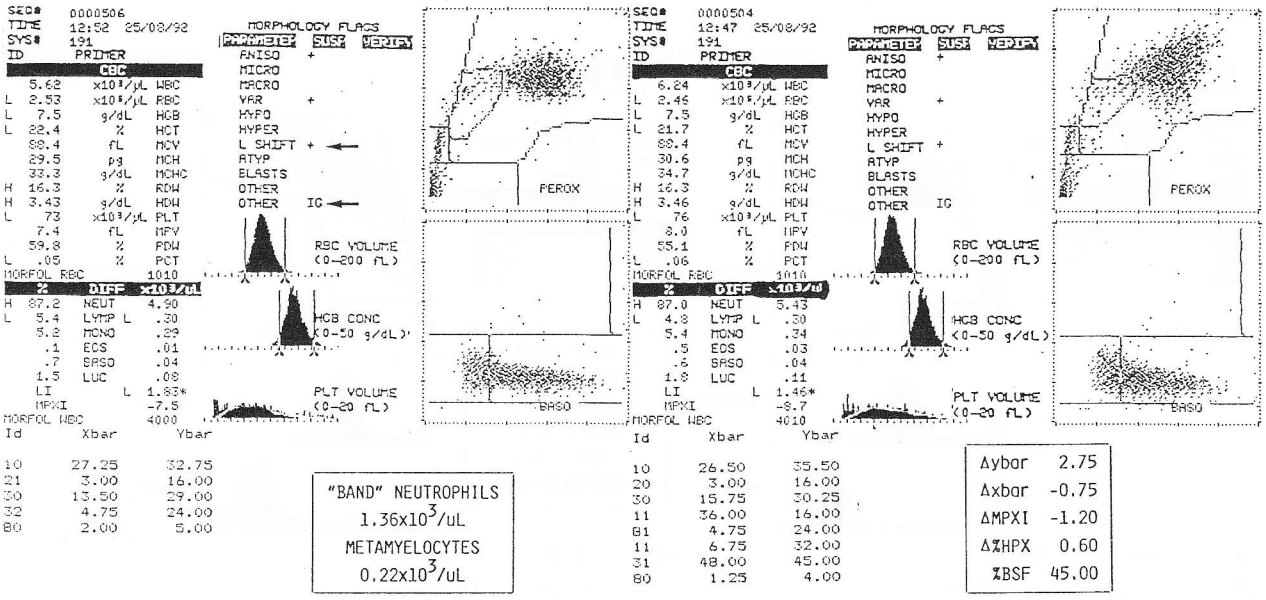


Fig. 1 H*2 Report 1 and Research Screen 2 from a normal blood sample before (left) and after (right) FMLP treatment. PMN morphological changes (scatter increase) were measured by variation of ybar values at Id 10 on report Research Screen 2. FMLP activation of the neutrophil-induced MPO depletion (decreased xbar and MPXI) and microscopically demonstrable PMN bipolar shape formation (BSF).
Non standard abbreviations: CBC = Complete Blood Count; DIFF = Differential WBC count; LUC = Large Unstained Cells; LI = Lobularity Index; MPXI = Mean Perox Index; %HPX = High Peroxidase % cells; L and H = Low or High value; PEROX = Cell scatter (on y-axis) and absorbance of peroxidase cytochemical reaction (on x-axis) on PEROX channel; SUSP = Suspected morphology flags.
For further explanations, e. g. the abbreviations, ask the authors.

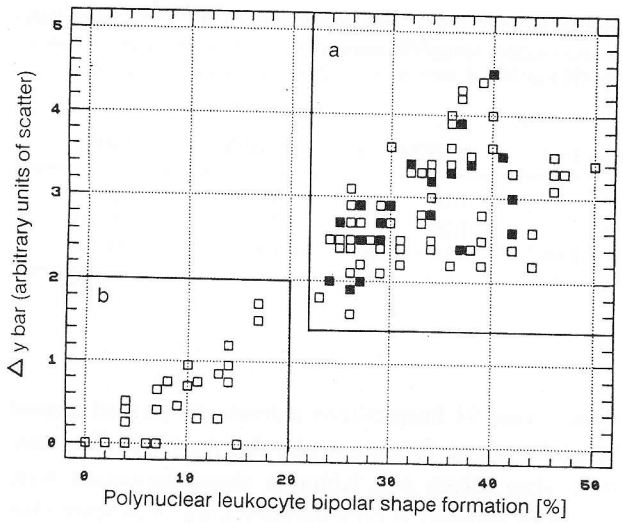


Fig. 2 Relationship between polymorphonuclear leukocyte bipolar shape formation and scatter rise (△ybar) after formyl-methionyl-leucyl-phenylalanine activation in blood samples from 20 healthy subjects (■) and 91 hospitalized patients (□). At ybar basal values ⩾ 37.00 arbitrary units of resting neutrophils (Box b), themorphological response (both instrumental and microscopic) was remarkably defective or absent. Box a includes normal samples and samples from hospitalized patients with ybar basal values < 37.00 arbitrary units.

oxidase depletion of formyl-methionyl-leucyl-phenylalanine-activated neutrophils in a number of cases, whereas high peroxidase cells were only barely increased, confirming abnormal myeloperoxidase distribution rather than increased enzymatic activity (5). The incubation of normal samples with lipopolysaccharide induced an increase in polymorphonuclear leukocyte scatter values at concentrations > 5 μg/l, with a higher response to formyl-methionyl-leucyl-phenylalanine at low lipopolysaccharide concentration and a null response at lipopolysaccharide concentrations ⩾ 50 μg/l.

Discussion

By analysis of polymorphonuclear leukocyte sizing in the H*2, we were able to determine a volumetric polymorphonuclear leukocyte threshold (⩾ 37.00 arbitrary units of ybar) over which neutrophils constantly showed a defective morphological response to formyl-methionyl-leucyl-phenylalanine in vitro. The close correlation between a large volume of resting neutrophils and decreased responsiveness to formyl-methionyl-leucyl-phenylalanine raises the question of the cause of this observation. The physical behaviour, as well as the defective formyl-methionyl-leucyl-phenylalanine response, may resemble a morphological and functional state of young, immature neutrophils, but "polymorphonuclear leukocyte volume does not correlate with polymorphonuclear leukocyte age" (6). Furthermore, chemotaxis in "band" neutrophils, reflecting the direct migration of cells in a gradient of chemoattractant, does occur, although it is decreased and approximates to half

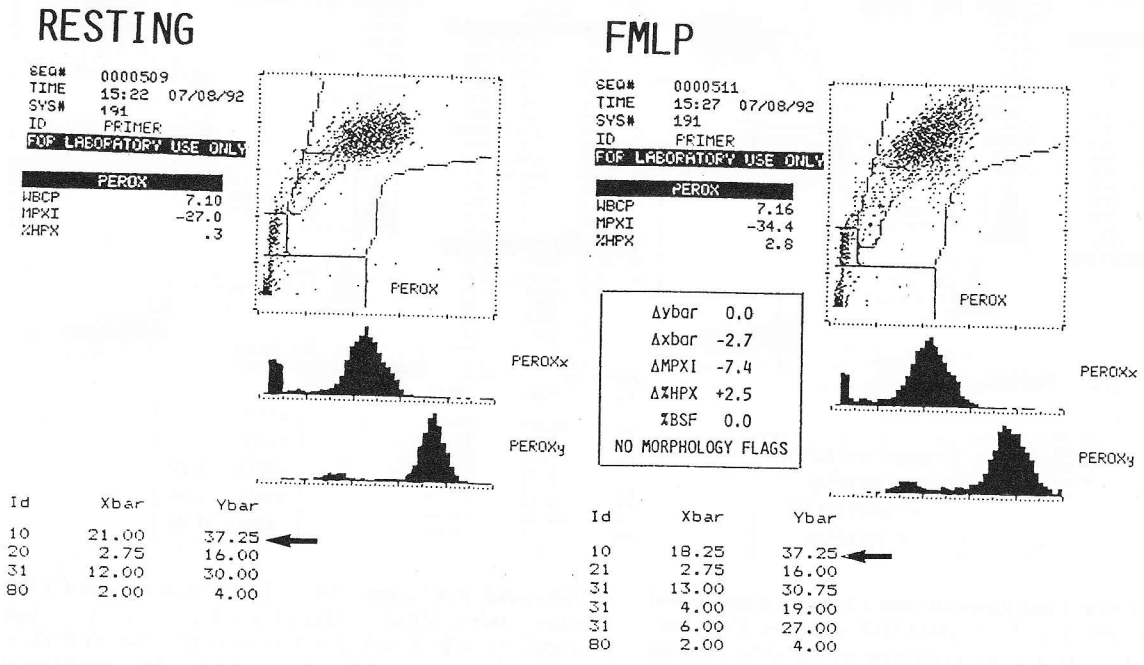
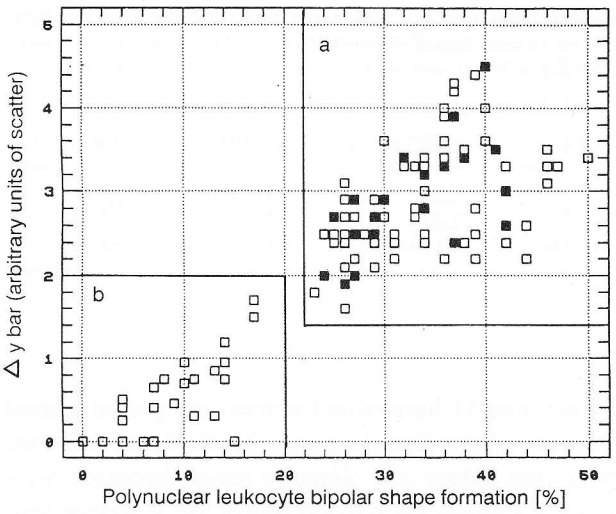


Fig. 3 Instrumental data in a representative case of polymorphonuclear leukocyte unresponsiveness to formyl-methionyl-leucyl-phenylalanine. Polymorphonuclear leukocyte scatter values (ybar) were unchanged (arrows), whereas the myeloperoxidase index (xbar and mean peroxidase index) demonstrated myeloperoxidase depletion in formyl-methionyl-leucyl-phenylalanine-activated neutrophils. Microscopic examination showed no polymorphonuclear leukocyte bipolar shape formation (BSF).



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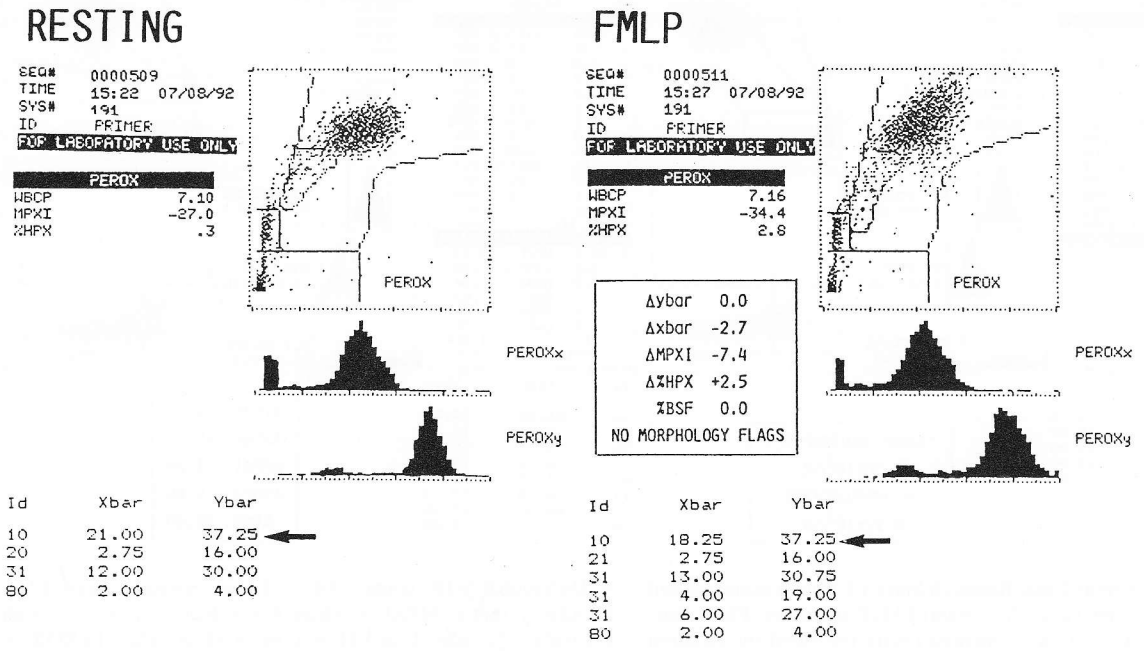

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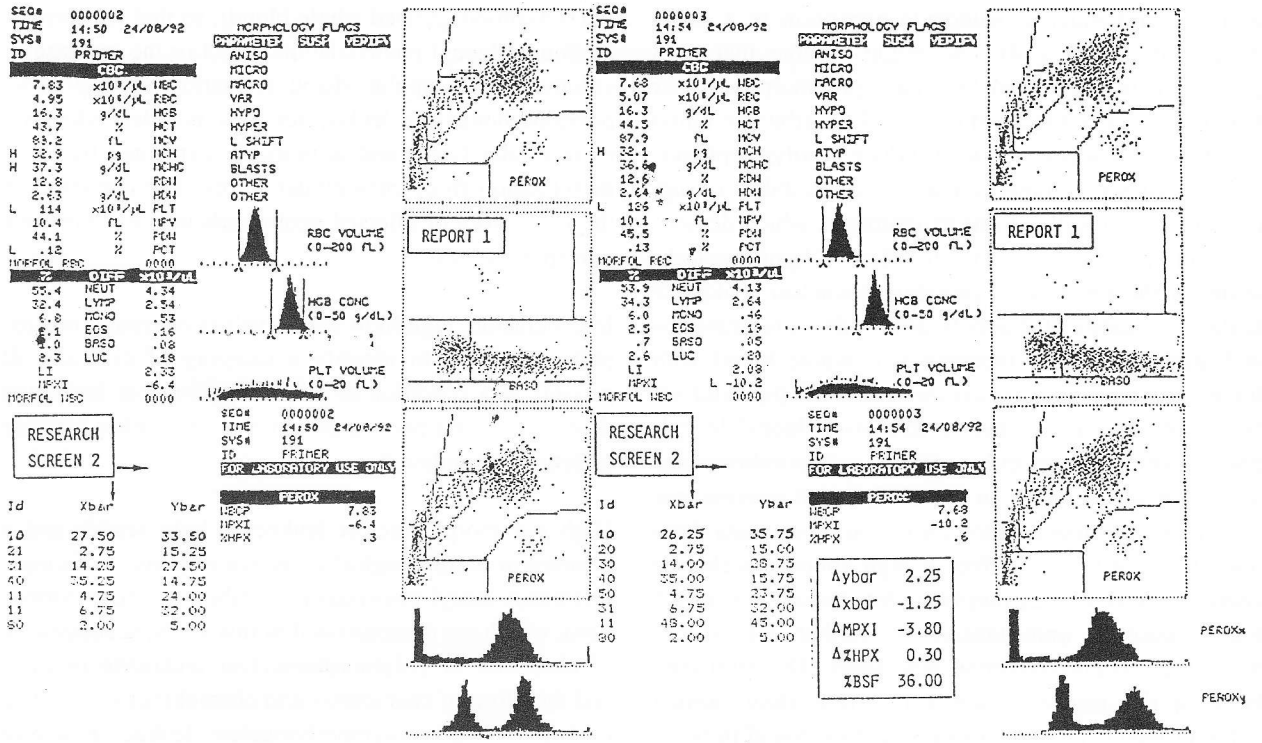

Fig. 4 H*2 analysis of a blood sample containing "band" neutrophils and metamyelocytes (arrows on morphology flags) before (left) and after (right) formyl-methionyl-leucyl-phenylalanine activation. Polymorphonuclear leukocyte morphological response to formyl-methionyl-leucyl-phenylalanine, both instrumental (ybar values) and microscopic (%bipolar shape formation), was plainly shown and similar to the controls.

the activity of segmented neutrophils (7). Indeed, normal or increased values for volume and %bipolar shape formation were seen after formyl-methionyl-leucyl-phenylalanine stimulation in blood samples containing "band" neutrophils and also metamyelocytes (fig. 4). Conversely, our volume assay of formyl-methionyl-leucyl-phenylalanine-activated neutrophils, did show a formyl-methionyl-leucyl-phenylalanine volumetric rise as an effect of the stimulation. The same has been shown for polymorphonuclear leukocytes activated in vitro, by our group and by other authors using different stimuli (8−11). The causes of in vivo activation, inducing large polymorphonuclear leukocytes with ybar values $\geqslant$ 37.00 arbitrary units, may be investigated by analysis of pathogenetic events occurring in patients with high ybar basal values and defective or null response to formyl-methionyl-leucyl-phenylalanine.

Of 24 adult hospitalized patients with polymorphonuclear leukocyte ybar basal values $\geqslant$ 37.00 arbitrary units, showing no response or a defective response to formyl-methionyl-leucyl-phenylalanine, 18 were males and 6 females. Fourteen patients were in intensive care units. Nine males and four women had sepsis with blood cultures positive for *Gram*-negative organisms. Six male patients with severe sepsis and *Gram*-negative bacteraemia had septic shock (sepsis associated to hypotension and organ dysfunction). Two males and a woman suffered from non-bacteraemic infections after burn injury. Two other patients, a male and a woman, were admitted into hospital with fever and cachexia in advanced neoplastic disease. Since all clinical and laboratory features found in patients with polymorphonuclear leukocyte ybar basal values $\geqslant$ 37.00 arbitrary units may be included in the "Systemic Inflammatory Response Syndrome" (SIRS) (12), we analysed the incidence of this syndrome among the other 67 patients with polymorphonuclear leukocyte ybar basal values $<$ 37.00 arbitrary units. The diseases of these patients did not include neoplasms of B-, T- and histiocytic/reticulum-cell origin. The result was that 8 patients affected by SIRS did not show an alteration in polymorphonuclear leukocyte basal size or response to formyl-methionyl-leucyl-phenylalanine, which represents 8.8% false negative results for this "diagnostic test" for SIRS. Therefore, the predictive value of the positive test was 100%, whereas the predictive value of the negative test was 90%, demonstrating excellent specificity and poor sensitivity (75%). Since numerous characteristic events of SIRS are related to the effects of endotoxin and other inflammatory mediators (13) and endotoxin is a clinically important mediator

of polymorphonuclear leukocyte activation in a dose-dependent fashion (14), one might assume that in 8 patients with SIRS and "normal" polymorphonuclear leukocyte scatter basal values (< 37.00 arbitrary units) endotoxaemia was too low to induce a polymorphonuclear leukocyte volumetric rise. This possibility is supported by the results of our experiments, which demonstrate in vitro a dose-dependent effect of lipopolysaccharide on the increase of polymorphonuclear leukocyte scatter (volume) at lipopolysaccharide concentrations ⩾ 5 μg/l. Only after incubation of whole blood with lipopolysaccharide at concentrations ⩾ 50 μg/l, did we fail to observe a response to formyl-methionyl-leucyl-phenylalanine, based either on the polymorphonuclear leukocyte scatter assay in the H*2, or on microscopic recognition of bipolar shape formation. These data demonstrate a deactivating effect of high lipopolysaccharide concentrations on polymorphonuclear leukocytes, which finally become unresponsive to further formyl-methionyl-leucyl-phenylalanine activation. The similarity between the results obtained by stimulating normal polymorphonuclear leukocytes and those found in polymorphonuclear leukocytes from SIRS patients suggests that neutrophils activated in vivo by endotoxin or other factors may also become unresponsive to chemotactic stimulation. Formyl-methionyl-leucyl-phenylalanine-responsive and -unresponsive polymorphonuclear leukocytes were stimulated in a Ca^{2+}/Mg^{2+}-free medium (EDTA-anticoagulated whole blood), so that the formyl-methionyl-leucyl-phenylalanine effect or the increase in volume and %bipolar shape formation in responsive polymorphonuclear leukocytes was not dependent on extracellular Ca^{2+}, and is probably explained by Ca^{2+} mobilization from intracellular stores. The depletion of the Ca^{2+} store of affected neutrophils may explain their unresponsiveness.

In conclusion, high light scatter values of resting neutrophils may help to identify a category of critically ill patients in a condition of polymorphonuclear leukocyte deactivation and poor responsiveness to further toxic or infectious stimulation.

High polymorphonuclear leukocyte light scatter and a concurrent morphological defective response to formyl-methionyl-leucyl-phenylalanine (%bipolar shape formation), which we demonstrated in this study, is suggestive of alteration of polymorphonuclear leukocyte motility and therefore of chemotaxis and chemokinesis (15). The finding of high polymorphonuclear leukocyte scatter with the H*2 may provide a useful screening test, so far unreported, for detecting a polymorphonuclear leukocyte functional anomaly. This new instrumental information of polymorphonuclear leukocyte scatter values ⩾ 37.00 arbitrary units should be considered significant, provided that the instrumental standardization of the

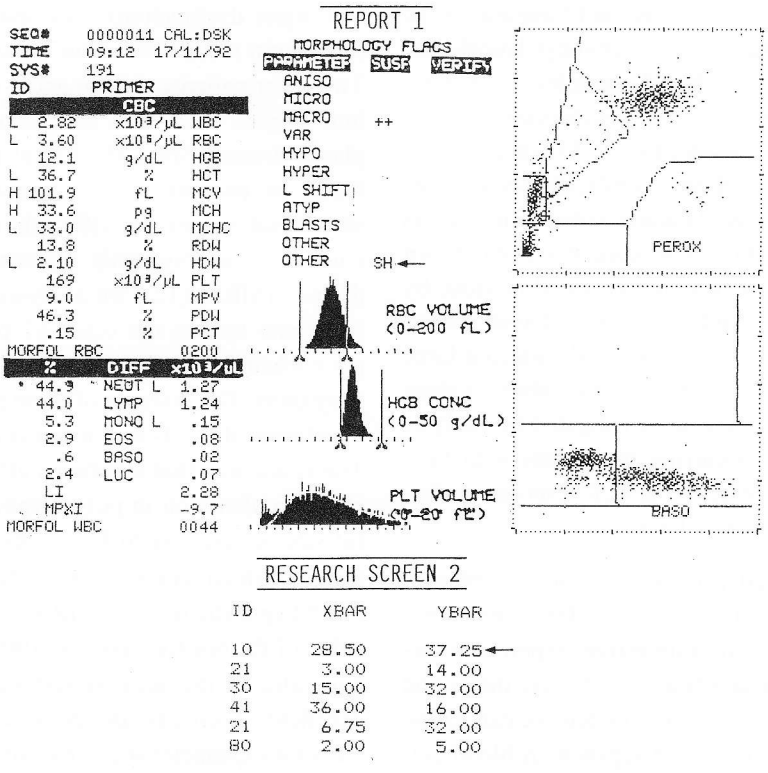

Fig. 5 The additional flag of "scatter high" (SH), on Report 1, indicates samples with neutrophil-scattered light ⩾ 37.00 arbitrary units of ybar value printed on Research Screen 2.

peroxidase optic is adjusted at Ky 90 ± 5 gain factor and that blood samples are processed no more than 3 h following venipuncture. A recent software implementation of our H*2 allows identification by flag SH ("Scatter High") on Report 1, for samples with ybar value ⩾ 37.00 arbitrary units (fig. 5). The flag makes the screening easier, and no watching or printing of the Report Research 2 is needed.

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Prof. Ugo Lippi
Laboratorio di Chimica Clinica ed Ematologia
Ospedale Civile Maggiore
Piazzale Stefani, 1
I-37126 Verona
Italy