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ORIGINAL ARTICLE

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A simple method for measuring the F-actin content of human polymorphonuclear leukocytes in whole blood

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Abstract We have developed an improved method for measuring the filamentous (F) actin content of human blood polymorphonuclear leukocytes (PMNs). The essential feature of the method is the immediate fixation of the F-actin cytoskeleton. Fresh whole blood (100 µl) is shock-cooled by the addition of 1.0 ml of a mixture of 18.75% glycerol and 5% formaldehyde in phosphate buffer pre-cooled to –8°C and subsequently fixed at 4°C for 15 min. After lysis in distilled water and removal of the red blood cells by centrifugation, the F-actin cytoskeleton of the PMNs is stained with fluorescein isothiocyanate (FITC)-phalloidin and quantified by means of flow cytometry. In healthy test subjects, PMN stimulation by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) for 20 s resulted in a significantly increased F-actin assembly, while in patients with multiple organ failure, two subpopulations arose: one with greater F-actin content and a second with lower F-actin content in comparison with the unstimulated blood sample. This simple and fast method may be a useful tool in basic and clinical research.

Keywords Polymorphonuclear leukocytes · PMN · Cytoskeleton · Actin · FMLP

Introduction

The characteristic functional features of polymorphonuclear leukocytes (PMNs) are locomotion, adhesion, phagocytosis, and granule release, all essentially dependent on remodeling processes between monomeric globular (G) and polymeric, filamentous (F) actin [3, 12, 24]. Moreover, the polymerization degree of the actin cytoskeleton is a major factor modulating cell viscosity and rigidity [15, 20, 27]. Increased rigidity of circulating PMNs, in turn, is supposed to contribute to such current problems in medicine as high blood pressure with atherosclerosis and diabetes [10. 17, 18, 21] and leukostasis during shock, and multiple organ failure [2, 22, 25]. Pentoxyphylline is widely used to increase blood flow in atherosclerosis and is thought to act mainly via depolymerization of the actin cytoskeleton [1, 7, 8, 9, 19, 25].

In contrast to the outstanding practical importance of this topic, extensive investigations of the role of actin remodeling in diseases are rare and contradictory. A main reason is the lack of appropriate methods that (1) are simple, while accurate enough for a broad use, and (2) preserve F-actin in or near the genuine polymerization state reflecting the actual situation in the circulating blood. The methods commonly used require PMNs isolated from the whole blood. Cell separation not only renders the laboratory handling time-, labor- and cost-intensive but, just as disadvantageously, activates the sensitive PMNs [4] that are able to remodel the actin cytoskeleton within a few seconds after stimulation [10].

We have developed an improved method for the demonstration of F actin in human PMNs that avoids these shortcomings. The novel and outstanding trait of the method is that the actual state of the PMN cytoskeleton in the fresh, whole blood is fixed within fractions of seconds. The fixed PMNs are separated from the red blood cells in a subsequent step. The F-actin staining and the evaluation follow established methods. The method is fast, easy to handle, inexpensive, and yields well-reproducible results.

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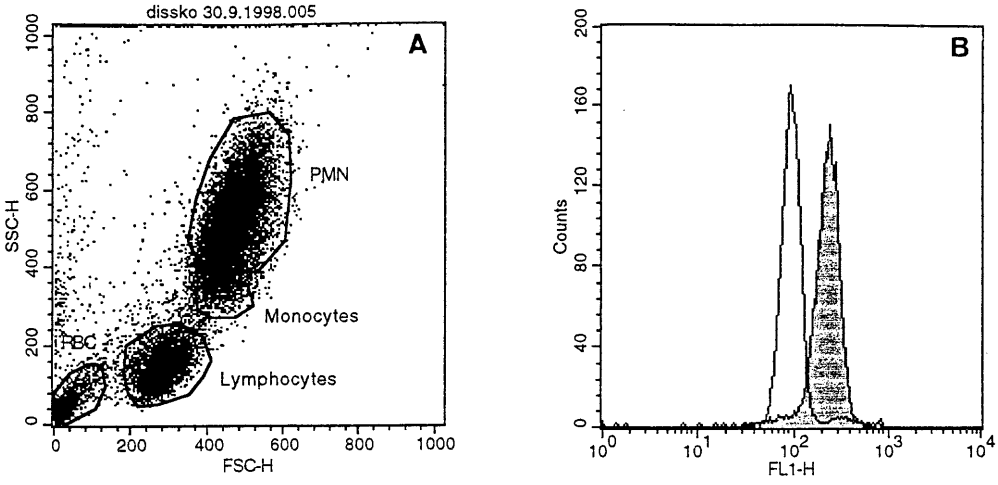
Materials and methods

The essential traits of the method are paralysis of the cell metabolism by shock-cooling the blood sample to the freezing point, the subsequent fixation of the blood cells at 4°C, the removal of the red blood cells by osmotic lysis, and the staining of the F actin in the remaining white cells with fluorescein isothiocyanate (FITC)-phalloidin.



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	8502	100.00	55.33	95.89	91.82	41.44	90.58	89

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	8770	103.15	57.07	223.92	215.47	26.33	222.67	232

Fig. 1 Example of the F-actin content in the blood polymorphonuclear leukocytes (PMNs) of a healthy test person. **A** The linearly scaled dual-parameter light scatter diagram of the unstimulated (*blank*) blood sample shows the lymphocyte, monocyte, and granulocyte populations. The lysed red blood cells were discarded with the supernatants, and only slight debris is left (*lower left corner*). **B** The log-scaled fluorescence of the fluorescein isothiocyanate (FITC) phalloidin-marked F actin of the cells in the granulocyte gate in **A**. The histogram statistics below the diagrams represent numerical fluorescence data. The *empty peak* and the *upper data line* refer to the blank, and the *shaded peak* and the *lower data line* refer to the reaction to formyl-methionyl-leucyl-phenylalanine (FMLP). Under both unstimulated and stimulated conditions, the peaks were narrow and clearly distinct. However, FMLP stimulation caused an increase in F actin (means and peak channel values). *SSC-H* side scatter; *FSC-H* forward scatter; *FL1-H* log fluorescence intensity

Blood was gently withdrawn from a cubital vein in ammonia-heparin S-Monovette syringes (Sarstedt, Nümbrecht, Germany) and processed for the F-actin check within 20 min. [4]. Vacuum systems are not suitable since they activate the PMNs. The blood was pipetted into mictotubes (no. 72.690, Sarstedt) in 100-µl portions.

Actin polymerization was stimulated by adding 100 µl of a 1.5 µM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) solution (Sigma-Aldrich, Vienna, Austria, cat no. F3506) in Hanks balanced salt solution [26] pre-warmed to 37°C to 100 µl of whole blood. After 20 s, PMN activation was stopped by fixation. Unstimulated blood samples (blanks) were checked in parallel. Both stimulated and unstimulated samples were tested in duplicate; the whole test thus was comprised of 400 µl whole blood.

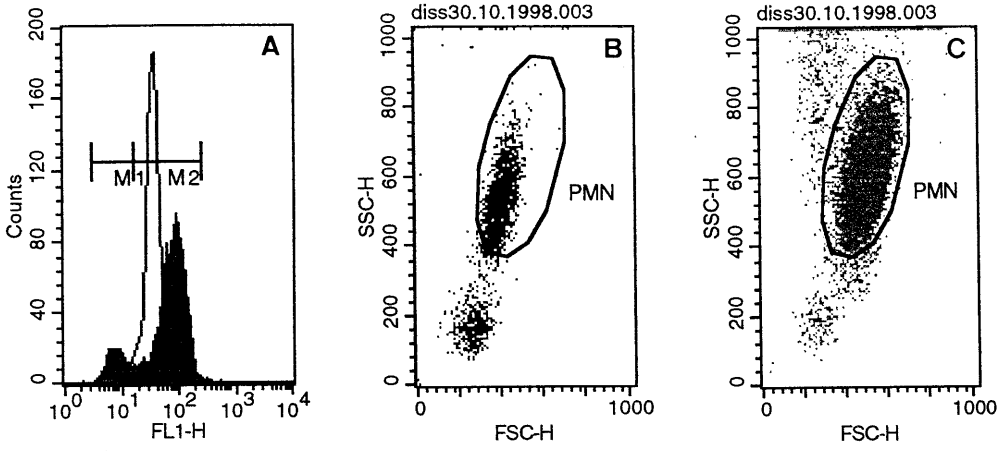
The mixture used for fixation consisted of two parts of double-concentrated phosphate buffer, pH 7.4, one part of 20% formaldehyde, prepared from paraformaldehyde, and one part of 75% glycerol in water. Glycerol addition lowers the freezing point and accelerates red blood cell lysis. For fixation, 1 ml of the fixation mixture precooled to –8°C was pipetted on a 100-µl blank sample and on a 200-µl FMLP sample. The samples were fixed at 4°C for 10 min. and subsequently centrifuged for 5 min at 4°C and 200 *g*. The supernatants were discarded.

The pellets were resuspended in 1 ml of distilled water, and the red blood cells were lysed at room temperature for 20 min, centrifuged for 5 min at 200 *g*, and the supernatants containing the remnants of the red blood cells were discarded. To prevent impairment of erythrocyte lysis, the whole fixation time should not exceed 20 min. However, blood samples can be stored for several hours when frozen below –20°C immediately after addition of the fixation mixture. After thawing, the samples can be processed. Since the fixation time influences FITC-phalloidin binding, fixation conditions should be constant.

The specificity of phalloidin binding to F actin is well established. We essentially followed the methods of Wallace et al. [26] and Sklar et al. [23]. A 3.3-µg/ml methanol FITC-phalloidin (Sigma-Aldrich, Vienna, Austria, cat no. P5282) stock solution was prepared and stored at –80°C in the dark. For F-actin staining, an instant solution was freshly prepared by mixing one part of the stock solution with nine parts of distilled water. The fixed white cells in the pellets were suspended in 350 µl of the FITC-phalloidin instant solution, stained in the dark at room temperature for 30 min, and centrifuged as above. Finally, the cells were resuspended in 1 ml of FACS FLOW (Becton Dickinson, San Jose, Calif.), and their fluorescence was measured in a FACSCAN Becton Dickinson flow cytometer at 488 nm wavelength. The PMN population could be easily identified on the dual-parameter light scatter diagram (Fig. 1A). With the gate set for the PMNs, the log-scaled fluorescence histogram from 10.000 PMNs was determined. The average F-actin content of a population was expressed as the mean of the fluorescence intensity. The scatter of the F-actin content within a population was estimated by the coefficient of variance (CV) of the histogram.

Ten male and ten female healthy test subjects, aged 33.2±9.6 years (mean±SD) and 29.0±6.1 years, respectively, were tested to outline the normal range of the F-actin contents in unstimulated PMNs and after FMLP stimulation. Blood was withdrawn with informed consent. To illustrate the practical usefulness of the method, the F-actin reactivity of a patient with multiple organ failure (a participant in an ongoing study) is presented.

The means of the duplicate samples were taken for statistic calculations. Differences between the values of males and females were compared using the Mann-Whitney test, and differences between the FMLP and blank values of the means and the CV were compared using the Wilcoxon test. Statistical values were expressed as mean±SD.



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10047	100.00	67.90	33.93	32.55	29.17	33.38	35
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10030	99.83	67.79	69.20	53.82	55.06	69.78	80
M1	3, 16	1298	12.92	8.77	8.65	8.21	33.21	8.06	6
M2	16, 255	8725	86.84	58.97	77.98	71.11	40.30	75.67	80

Fig. 2 A patient with multiple organ failure. **A** The log-scaled fluorescence of the unstimulated blood sample (*empty peak*) and after formyl-methionyl-leucyl-phenylalanine (FMLP) stimulation (*shaded peaks*). While the peak of the unstimulated blood is narrow and distinct (*upper data line*), polymorphonuclear leukocytes (PMNs) under FMLP stimulation formed two subpopulations with an F-actin content lower (M1) and higher (M2) than in the unstimulated blood sample (*lower three data lines*). The quantitative relation of M1:M2 is 12.92%:86.84%. **B**, **C** The linearly scaled light scatter diagrams referring to the subpopulations M1 (**B**) and M2 (**C**), indicating that the population M1, with low F-actin contents, consisted of small PMNs with low granularity, while the population M2, with high F-actin contents, consisted of large PMNs with higher granularity

Results and discussion

Figure 1A, B represents the typical reaction pattern of a healthy test person. The dual-parameter light scatter diagram (Fig. 1A) shows the familiar three populations of leucocytes: lymphocytes, granulocytes and, in between, the monocytes. Most of the remnants of the lysed red blood cells were discarded with the supernatants. The F-actin contents in the PMNs of the unstimulated blood sample (empty peak, Fig. 1B) are rather uniform as expressed by the narrow peak of the log fluorescence intensity. When the PMNs in the same blood sample were stimulated by FMLP (shaded peak, Fig. 1B), F-actin formation was triggered, as indicated by the increased average fluorescence (means, peak channels).

Since no statistical differences between the means and the CV values of male and female test persons could be established, both genders were treated in a common group. The mean fluorescence values of the 20 blank and the FMLP-treated samples were 94.99±51.7 and 199.05±140.8, respectively (P<0.0001), and the CV values were 34.35±3.5 and 45.86±11.42, respectively (P=0.0003).

Figure 2A–C shows the reactivity of the actin cytoskeleton in a patient with multiple organ failure. The unstimulated PMNs had low but uniform F-actin contents as expressed by the narrow peak (Fig. 2A, empty peak). In contrast, FMLP stimulation triggered the formation of two populations with distinctly different F-actin conformations, which are marked in Fig. 2A as “M1” and “M2”. The high CV value reflects this situation. In the dual-parameter scatter plot, the low F-actin (M1) population exhibited a low forward scatter (FSC) and sideward scatter (SSC), meaning small PMNs with sparse granularity (Fig. 2B), and the high F-actin (M2) population exhibited a comparably high FSC and SSC, reflecting larger PMNs with ample granules (Fig. 2C). These differences could also be illustrated by plotting florescence against SSC and FSC, respectively (Fig. 3A, B). Priming of a PMN subpopulation toward FMLP stimulation could also be induced by tumor necrosis factor (TNF)α in vitro [5, 6, 11]. Since degranulation requires the depolymerization of F actin [13, 14], the M1 subpopulation can be supposed to consist of highly sensitive PMNs that have released their granules within the period of 20 s upon FMLP stimulation in vitro, resulting in a reduction of cell size (FSC) and granularity (SSC).

Conformational changes of the blood PMN actin cytoskeleton upon stimulation reflect the inflammatory state of an organism and therefore may be useful in the definition and diagnosis of inflammatory diseases and in therapy monitoring. The method is easy to handle and may be applicable to a variety of cell types and topics in research and in clinical fields.

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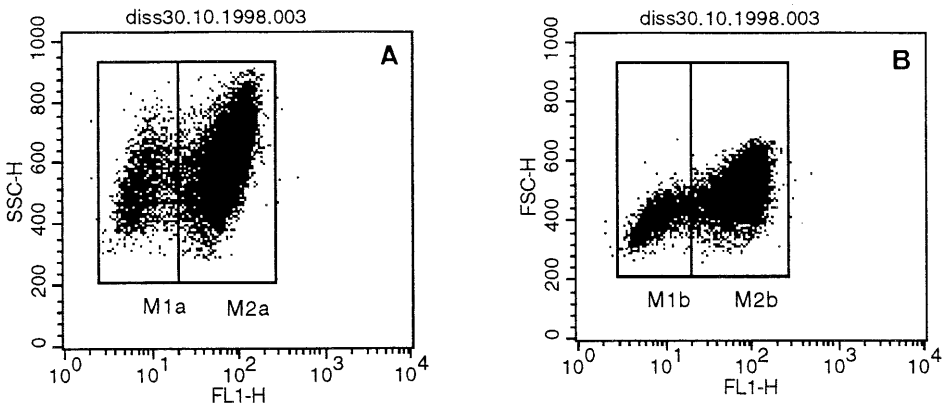

Fig. 3 Data of the patient shown in Fig. 2, but fluorescence plotted against the side scatter (SSC; **A**) and the forward scatter (FSC; **B**). The gates M1a and M1b represent the M1 (low fluorescence), and the gates M2a and M2b represent the M2 (high fluorescence) populations in Fig. 2A. Low fluorescence corresponds with low granularity and small cell size

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
M1a	1810	16.59	14.34	9.14	8.38	403.45	400.70
M2a	9093	83.36	72.02	76.96	70.59	498.17	494.37
M1b	1808	16.57	14.32	9.15	8.39	403.56	400.82
M2b	9093	83.36	72.02	76.96	70.59	498.17	494.37

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