

The application of a flow cytometric assay for evaluation of phagocytosis of neutrophils

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Phagocytosis and the release of oxidative products generated by the respiratory burst have been studied *in vitro* under the influence of non-steroidal anti-inflammatory drugs: naproxen and ibuprofen, using phagocytes of peripheral blood from healthy human donors. Phagocytosis was monitored by flow cytometry in order to investigate the uptake of propidium iodide-labelled bacteria (*Staphylococcus aureus*) by polymorphonuclear leucocytes. In addition, the phagocytic capacity and percentage of killed bacteria was measured in isolated neutrophils using the Pantazis & Kniker method. It was found that naproxen and ibuprofen affect the phagocytic function and hydrogen peroxide production in the examined granulocytes.

These methods might be useful in investigations on neutrophil functions.

The oxygen-dependent and oxygen-independent systems in phagocytic neutrophils play a crucial role in killing of bacteria during infection. Many methods are in use for evaluation of the phagocytic activity of polymorphonuclear leucocytes (PMNs), among others measurement of chemiluminescence of stimulated neutrophils [1]. Colorimetric methods for determination of reactive forms of oxygen are also applied [2].

In this study we tried to adapt the flow cytometric dichlorofluorescein (DCFH)-oxidation assay for estimation of the effect of non-steroidal anti-inflammatory drugs (NSAIDs): naproxen and ibuprofen on phagocytosis and bactericidal ability of neutrophils. The influence of NSAIDs on activated granulocytes is one of the factors of their anti-inflammatory action, especially in those

diseases in which phagocytosis plays a significant role in pathogenesis [1].

MATERIALS AND METHODS

Subjects. Heparinized (10 IU/ml) peripheral blood was obtained from healthy adult volunteers. Neutrophils were isolated from the blood after centrifugation in Gradisol G (Polfa, Kutno, Poland) gradient.

Non-steroidal anti-inflammatory drugs: naproxen: (*S*)-6-methoxy- α -methyl-2-naphthalene acetate (Syntex, Basel, Switzerland) was used at concentrations of 33 ng/ml and 66 ng/ml, and ibuprofen: α -(4-isobutylphenyl)-propionate (Polfa, Poland) at concentrations of 5.71 μ g/ml, 11.42 μ g/ml and 17.13 μ g/ml.

Abbreviations: DCFH-DA, 2',7'-dichlorofluorescein diacetate; NSAID, non-steroidal anti-inflammatory drug; PMN, polymorphonuclear leucocytes; PI, propidium iodide.

Reagents. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Acros (U.S.A.). It was dissolved in ethanol at a concentration of 25 mmol/l and stored in the dark at 4°C. Phosphate buffered saline (PBS) was from Biomed (Lublin, Poland).

Bacteria [8]. *Staphylococcus aureus*, strain ATCC 25923 were heat-killed at 60°C for 30 min and labeled with a 5% solution of propidium iodide (PI) (Sigma Chemical Co.) in normal saline solution for 30 min at room temperature in the dark. The fluorescent bacteria were washed three times in normal saline solution and suspended in calcium-free and magnesium-free PBS. The bacterial density was adjusted to an absorbance value of 2.5 at 620 nm. The number of bacteria at this density was about 2.4×10^9 colony-forming units/ml.

Simultaneous measurement of phagocytosis and production of hydrogen peroxide. A mixture of 50 µl of heparinized whole blood, 50 µl of PI-labeled *S. aureus* and 100 µl of 0.3 mmol/l DCFH-DA in PBS with or without 20 µl naproxen or ibuprofen was incubated for 30 min at 37°C. Then 1 ml of 3 mmol/l EDTA was added to terminate phagocytosis and prevent bacterial adhesion to the PMN membrane. After centrifugation, the erythrocytes were lysed by adding 1 ml of lysing solution (Ortho-mune). After 10 min intracellular PI and DCF fluorescence of PMNs were determined by flow cytometry (Cytoron Absolute, Ortho) at 488 nm excitation wavelength. When cells entered the laser beam, their dimension was measured as forward scatter light dispersion (FW-SC) and their granules were measured as right-angle scatter light dispersion (RT-SC). Additionally, the intensity of green, orange and red fluorescence was registered. The intensity of photoelectric signals was converted into numerical values (channels). The DCF fluorescence was measured in the green light beam (515–548 nm) and the PI fluorescence was registered in the red (over 607 nm) light, after logarithmic strengthening of the signal. The fluorescence intensity was expressed as the value of the "mean channel", calculated by ImmunoCount 2 software.

Evaluation of phagocytosis. To assess phagocytosis and bactericidal capability of

PMNs we used the modified method of Pantazis & Kniker [3]. The use of acridine orange (Sigma Chemical Co.) as described in the original method enables to distinguish the living from the dead bacteria inside a phagocyte. Thus, basing on this method, phagocytosis and bactericidal activity of neutrophils were registered simultaneously. Neutrophils isolated from heparinized blood were incubated with naproxen or ibuprofen for 30 min at 37°C and then a monolayer of neutrophils was prepared by dripping onto coverslips. The monolayer of adherent cells was incubated with standardized suspensions of bacteria for 30 min, then briefly stained with diluted acridine orange and examined under ultraviolet light. The following parameters were analyzed:

- 1) phagocytic capacity expressed as the mean number of bacteria ingested by 100 neutrophils
- 2) percentage of killed bacteria referred to the number of ingested bacteria.

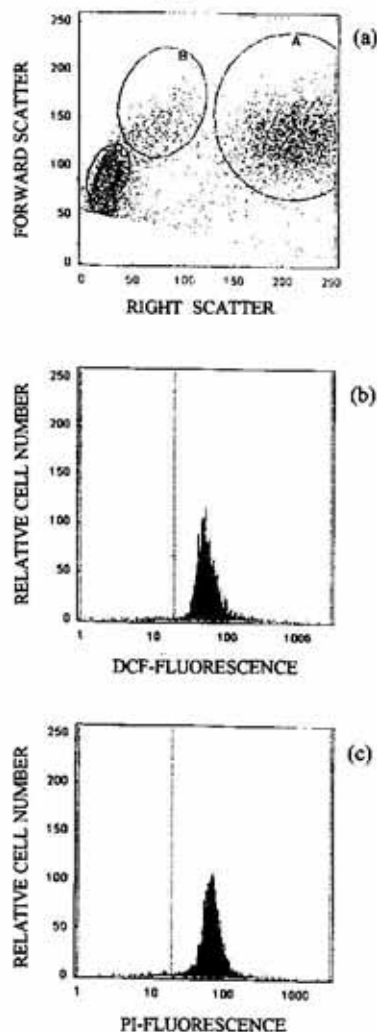
Statistical analysis. Significance of the differences at $P < 0.05$ was calculated using the paired *t*-test and referred to control values.

RESULTS AND DISCUSSION

Since the report of Vane [4] it has been known that non-steroidal anti-inflammatory drugs inhibit prostanoid production in human tissue. Recently, Mitchell *et al.* (cf. [5]) have demonstrated the ability of NSAIDs to inhibit in a different manner constitutive and inducible cyclo-oxygenases [5]. However, it has been suggested that inhibition of prostanoid production itself is not sufficient to account for the anti-inflammatory activities of NSAIDs [6]. According to an alternative hypothesis, inhibition of leucocyte functions does not involve inhibitory effects on prostaglandin biosynthesis [6].

In our study we have used the flow cytometric dichlorofluorescein (DCFH)-oxidation method, which was developed by Bass *et al.* [7] for quantitating the intracellular respiratory burst in a single cell and which avoids PMN activation due to isolation procedures. Oxidation of DCFH to DCF was catalyzed by

the oxidative neutrophil products within a cell during the respiratory burst stimulated by phagocytized PI-labelled *S. aureus*. By means of flow cytometry we have measured green-DCF fluorescence emission together with red-fluorescence, which was emitted by the ingested fluoresceinated bacteria (Fig. 1). The fluorescence intensity was proportional to the number of bacteria ingested. This two-color cellular staining permits measurement of two different functions of neutrophils in one step and thus could be of use for determination of the interactions between neutrophils and bacteria [8].



Phagocytic and respiratory burst activity of PMN incubated for 30 min with either naproxen or ibuprofen at concentrations used in clinical practice were determined. Using the flow cytometry phagocytosis test on whole blood it was found that naproxen and ibuprofen at higher concentration decreased the intensity of phagocytosis (Fig. 2). The

mean peak channel for naproxen was 57.8 compared with control value (without drug) of 78.4 and for ibuprofen mean peak channel was 57.8 ($P < 0.05$). At lower concentrations of the drugs the fluorescence intensity of phagocytes, which reflects the number of ingested bacteria, was found to be insignificantly lower than in controls.

The results obtained in the flow cytofluorimetry assays were corroborated by the Pantazis' and Kniker's fluorochrome method with isolated neutrophils. Changes in phagocytic capacity and percentage of killed bacteria measured after Pantazis & Kniker [3] is

Figure 1. Fluorescence distribution of PMNs from a healthy donor.

a) Cytogram showing the forward angle light scatter (FW-SC = y axis) and right scatter (RT-SC = x axis) in the following groups of leucocytes: A, granulocytes; B, monocytes; C, lymphocytes. These cells populations are better distinguishable by their right scatter value than by the forward scatter; b) representative histogram of "green" fluorescence (DCF-fluorescence) of PMNs phagocytizing bacteria (data from cytogram gate A); c) representative histogram of "red" fluorescence from PI-labeled *S. aureus* ingested by granulocytes (data from cytogram gate A).

shown in Figs. 3 and 4. As can be seen naproxen and ibuprofen significantly influenced the phagocytic capacity at every concentration of the drugs ($P < 0.001$). However, the percentage of killed bacteria after incubation with the drugs was lower than in controls but the difference was not statistically significant. Our results support the

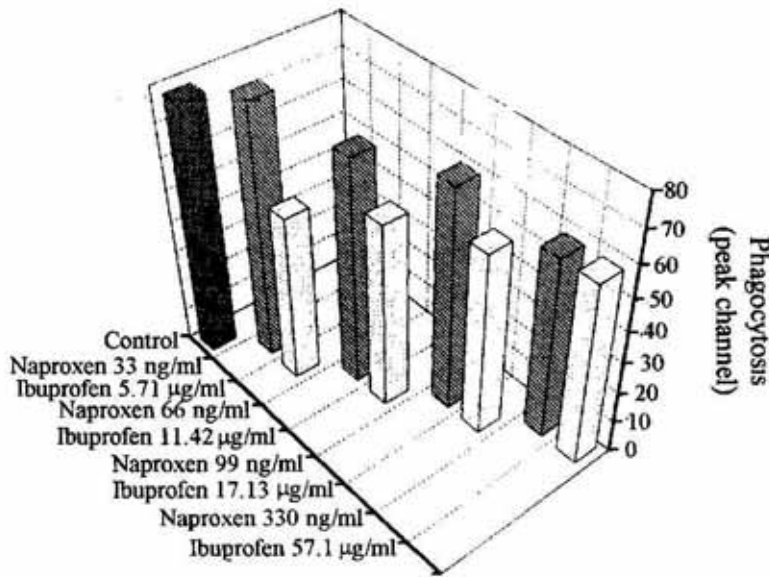


Figure 2. Effect of naproxen and ibuprofen on phagocytosis of neutrophils measured by flow cytometry.

The results are expressed as mean peak channel from 5 experiments.

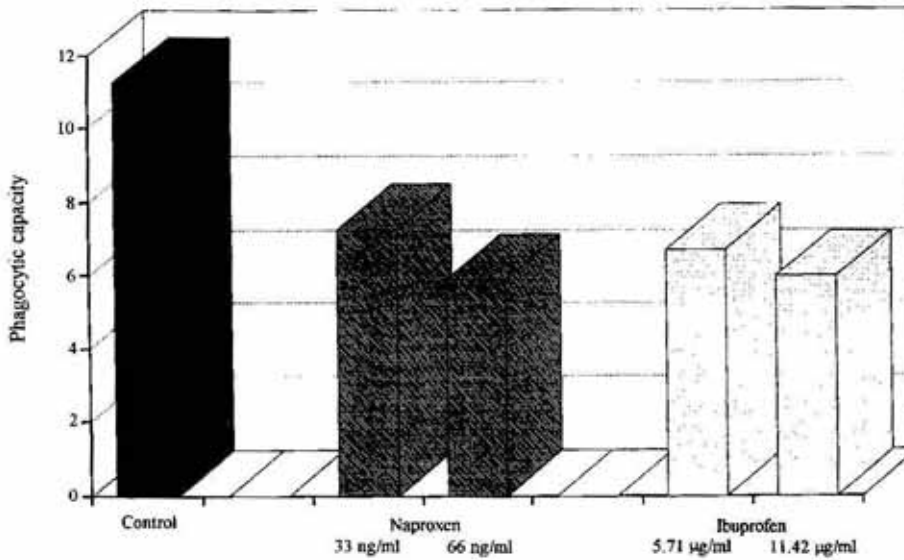


Figure 3. Phagocytic capacity, i.e. mean number of bacteria ingested by 100 neutrophils.

Values were measured according to Pantazis & Kniker's method [3] after incubation of neutrophils with naproxen or ibuprofen. The data represent means from 7 experiments, $P < 0.001$.

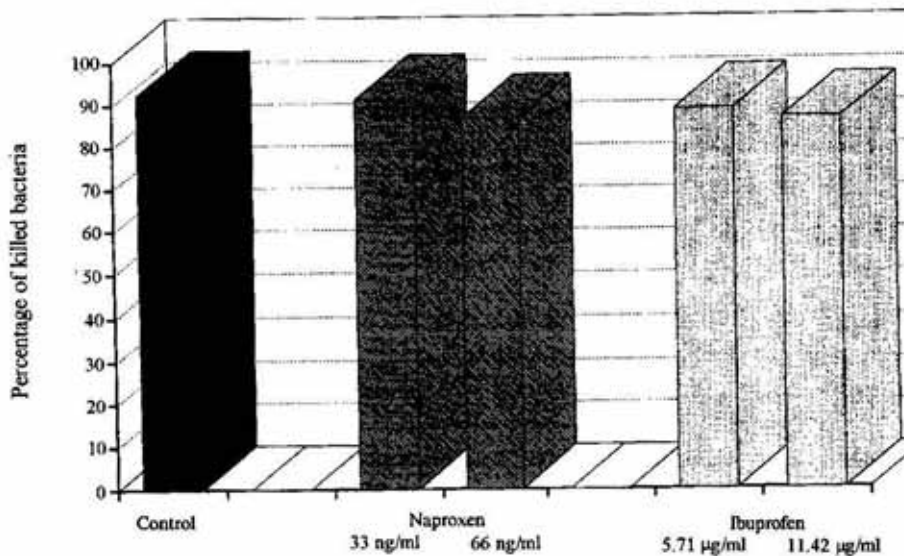


Figure 4. Percentage of killed bacteria, i.e. the number of killed bacteria referred to the number of ingested bacteria.

Values were measured after incubation of neutrophils with naproxen or ibuprofen. The data represent means from 7 experiments.

opinion that naproxen and ibuprofen suppress PMN functions.

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