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A simple assessment of human neutrophil adhesiveness

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Summary. The adhesiveness of human polymorphonuclear leukocytes was assessed in serum-coated polystyrene spectrophotometric cuvettes. Capped cuvettes, containing no more than 2×10^6 resting or concanavalin A-treated (100 µg/ml) polymorphonuclear leukocytes, were laid horizontally and subjected to three 90° rotations on their major axis at fixed times. After incubation at room temperature, non-adherent cells remaining in suspension were counted on the Coulter counter STKS hematological analyzer. After a 16-min incubation (4 min each side of the cuvette) the adhesion of concanavalin A-activated neutrophils ranged from 98% to 100% and the adhesion of resting neutrophils from 30% to 35% (mean 32.4±2.2%, $n=10$). An 8-min incubation (2 min each side) led to approximately 50% adhesion of concanavalin A-activated neutrophils (mean 49.9±2.2%, range 46%–54%, $n=16$), whereas the adhesion of resting cells was about 21% (mean 21.4±1.6%, range 19%–24%, $n=16$). The variation in percentage adhesion in repeated assays did not exceed 4% using concanavalin A-activated cells and 7.5% with resting neutrophils. The procedure is very rapid, easy to perform and precise, and no special apparatus or glassware is necessary. The method also allows microscopic evaluation of shape changes of adherent neutrophils through the clear sides of the cuvettes.

Key words: Neutrophils – Adhesion – Neutrophil adhesiveness

Introduction

The first response of polymorphonuclear leukocytes (PMN) to inflammation is adhesion to the endothelium of dilated capillaries and venules. The mechanism of PMN adhesion is under active investigation [5]. The discovery of monoclonal antibodies that bind to adhesion molecules [23] has provided new knowledge of PMN adhesion and allowed a breakthrough in the understanding of patients with abnormal phagocyte adherence and depressed inflammation [2, 7, 8, 22, 23].

The physiological study of PMN adhesiveness in vitro involves the assessment of PMN adhesion to a monolayer of endothelial cells [10]. Measurements of PMN adhesion to monolayers do not account for flow, which is obviously relevant to adhesion in vivo, and differences may exist in the properties of endothelial cells obtained from different tissues [10]. An alternative to these difficult techniques is to use monoclonal antibodies to measure the surface expression of the glycoprotein receptors involved in neutrophil adhesion [23]. However, adhesion may be independent of the expression of glycoproteins (integrins) that mediate cell adhesion [14] or may be due to interaction with ligands that are not members of the integrin family [24]. In addition, a circulating humoral factor (or factors), that modulates adherence of endothelium for PMN, has been described [1]. Lastly, the intrinsic PMN adhesiveness may be assayed in vitro by measuring the attachment to glass or plastic surfaces of resting or stimulated PMN, either separated or in whole blood [3–5, 9, 12, 13, 16–18, 21].

This paper describes a further simple technique for measuring in vitro the intrinsic adhesiveness of separated neutrophils. The assay takes no longer than 16 min, requires no special apparatus and glassware and the results are highly reproducible and in good agreement with those obtained by enzymatic assay of adherent cells as described elsewhere [3].

Materials and methods

Reagents. Ficoll (type 400) d1077 and 1119 containing sodium diatrizoate (Histopaque 1077 and 1119), phosphate-buffered saline (PBS, pH 7.2), concanavalin A (Con A) type IV from *Canavalia ensiformis*, *p*-nitrophenyl phosphate disodium hexahydrate (PNP) and Triton X-100 were purchased from Sigma (St. Louis, Mo., USA). Con A was dissolved in PBS (1.0 mg/ml) and used on the day of the experiment. PNP solution (10 mmol/l) was prepared with 50 mmol/l citrate buffer (pH 4.8) and stored at 4°C for not more

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than 1 week. Fetal bovine serum (FBS), (sterile, mitogen free) was from Bio-Division, Società Prodotti Antibiotici (Milan, Italy). Serum was inactivated by incubation at 56 °C for 45 min, divided into aliquots, stored at −20 °C and thawed before use.

Materials. PMN adhesion was measured in Macro type disposable polystyrene spectrophotometer cuvettes (10 × 10 × 45 mm, 2 clear sides) from KIMA (Padua, Italy). Suitable caps were made by cutting plastic needle stoppers of heparinized syringes in the Board Parker Preset 3CC kit from Becton Dickinson (Franklin lakes, N.J., USA). Seraclear filters were purchased from Technicon Instruments (N.Y., USA). Cell counting was performed on a Coulter counter STKS hematological analyzer (Coulter, Hialeah, Fla., USA) using 0.15 ml of sample.

Methods. Venous whole blood from healthy adult, non-smoking volunteers was collected in potassium EDTA (1.5 mg/ml). PMN were separated with a double discontinuous density gradient (Histopaque 1077-1119) according to the Sigma revised procedure. In order to achieve maximum neutrophil purity, only the central portion of the band at the 1077/1119 interface was collected after centrifugation. Separated PMN were washed three times with large volumes of PBS. Red blood cell contamination was excluded by hypotonic lysis: PMN pellets were treated with cold distilled water (2 ml) for 20 s, the same volume of double-concentrated PBS was then added to restore normal osmolality. After centrifugation, PMN pellets were gently resuspended in small volumes of PBS and suspensions were filtered through Seraclear. The device was thrust 3–4 cm into a polypropylene test tube (1.5 × 10 cm), suspensions were poured into Seraclear and filtered by slowly removing the device from the tube. With this procedure, fragments of unbroken pellets were eliminated and STKS obstructions during cell counting were avoided.

One-step discontinuous gradient separation of PMN yielded between 25% and 47% of neutrophils. The purity ranged from 96% to 99.7% and the viability of cells (trypan blue exclusion) always exceed 95%.

Neutrophils were resuspended in PBS at about 4×10^6/ml and two aliquots (0.45 ml) of suspension were processed in two FBS-coated cuvettes. Coating was performed by filling cuvettes with 50% FBS in PBS and, after standing for at least 2 h at room temperature, cuvettes were washed gently three times with PBS. Con A (0.05 ml) and the same volume of PBS were added to the test and control (resting PMN), respectively. Cuvettes were capped 3 mm deep with plastic caps and mixed; one of the longer sides was laid horizontally, so that the fluid at the bottom was uniformly distributed. Cuvettes were rotated at room temperature at fixed times, at 90° on the other three sides, and rested for the same interval, they were then stood up; the fluid at the bottom was collected with a plastic Pasteur pipette and then poured into siliconized glass tubes for PMN counting on STKS. PMN were counted twice and the mean value recorded.

Adhesion (%) was calculated using the following equation: 100 − number of non-adherent PMN/total PMN added to each cuvette × 100. Cuvettes containing adherent cells were washed three times with PBS, and PMN membrane acid phosphatase (ACP) was determined by adding PNP solution (1.0 ml) [3] containing 0.2% Triton X-100. The cuvettes were placed in a rotary mixer and incubated for 30 min at room temperature, so that the substrate solution was in repeated contact with the inside walls. The enzyme reaction was stopped with 0.5 ml of 2.0 M sodium hydroxide and the mixture poured into Eppendorf 1.5-ml Micro test tubes. Microtubes were centrifuged for 3 min in an Eppendorf centrifuge 5415C to avoid turbidity arising from the debris of Triton-lyzed cells. Absorbances were read at 405 nm in a Beckman DU-7 spectrophotometer against a reagent blank. The percentage of adherent cells was calculated from a standard curve obtained using known numbers of PMN (from 0.20 to 1.2×10^6). Direct microscopic examination of either living non-adherent or adherent PMN was performed using small drops of suspension or through the clear sides of the cuvettes, respectively.

Results

The percentage adhesion of resting of Con A-activated PMN after 8 and 16 min incubation are shown in Table 1. After a 16-min incubation (4 min each side of the cuvette), there were only a few (or no) non-adherent Con A-activated PMN and no cells were seen in suspension by microscopic examination. The mean adhesion was 99.1 ± 0.9% (n = 10). Adhesion of resting PMN ranged from 30% to 35% (mean 32.4 ± 2.2%; n = 10) with the same incubation time. After 8 min incubation (2 min each side), the mean adhesion of Con A-activated PMN was 49.90 ± 2.2% (n = 16) and of resting PMN 21.4 ± 1.6% (n = 16). No cell aggregates were observed by microscopic examination of non-adherent PMN remaining in suspension. A shorter incubation of 4 min (1 min each side) led to less adhesion (about 20%) of Con A-activated PMN and a negligible difference in the adherence of resting PMN. At an intermediate time of incubation (12 min, 3 min each side), microscopic examination of non-adherent PMN remaining in suspension demonstrated the presence of microaggregates which would lead to inaccurate cell counting.

When aliquots of the same PMN suspension were retested for adhesion, differences did not exceed 4% after an 8-min incubation of Con A-treated PMN and 2% after a 16-min incubation. Differences in the adhesion of resting PMN on retesting were not more than 7.5% at both incubation times.

Spectrophotometric assay of PMN adhesion, using the reference method [3], demonstrated a good correlation (P = 0.35) between percentage adhesion measured by the two methods (Fig. 1). The standard curve of ACP was linear within the fixed range of adherent PMN and thus the number of adherent cells ($\times 10^6$) was calculated by multiplying the optical density by 2.07. Since the PMN concentration in each cuvette did not exceed 2×10^6 cells, and after 8 min of incubation the adhesion was not more than 54%, the curve (upper limit 1.2×10^6 PMN) allowed the measurement of the highest possible number of adherent cells. The methods were not compared after a 16-min incubation, since with 98%–100% adhesion there were too few non-adherent cells in suspension.

Microscopic examination of adherent cells (Fig. 2) demonstrated an increase in adhesion due to Con A

Table 1. Percentage adhesion (mean ± SD and ranges) of resting and concanavalin A-activated polymorphonuclear leukocytes (PMN)

PMN treatment	Adhesion (%)	
	8-min incubation	16-min incubation
None (resting PMN)	21.4 ± 1.6 (19–24)	32.4 ± 2.2 (30–35)
n	16	10
Concanavalin A (100 μg/ml)	49.9 ± 2.2 (46–54)	99.1 ± 0.9 (98–100)
n	16	10

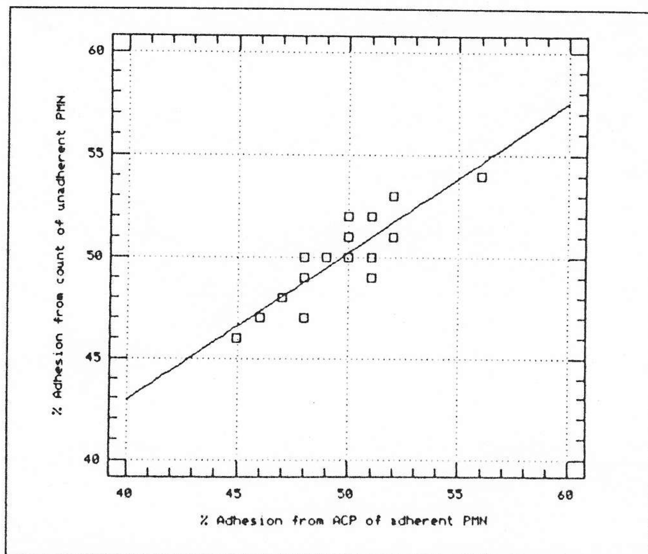

Fig. 1. Correlation between percentage adhesion of concanavalin A (Con A)-activated polymorphonuclear leukocytes (PMN) measured by counting non-adherent cells and by assay of membrane acid phosphatase (*ACP*) of adherent PMN; $r=0.87$, $n=16$

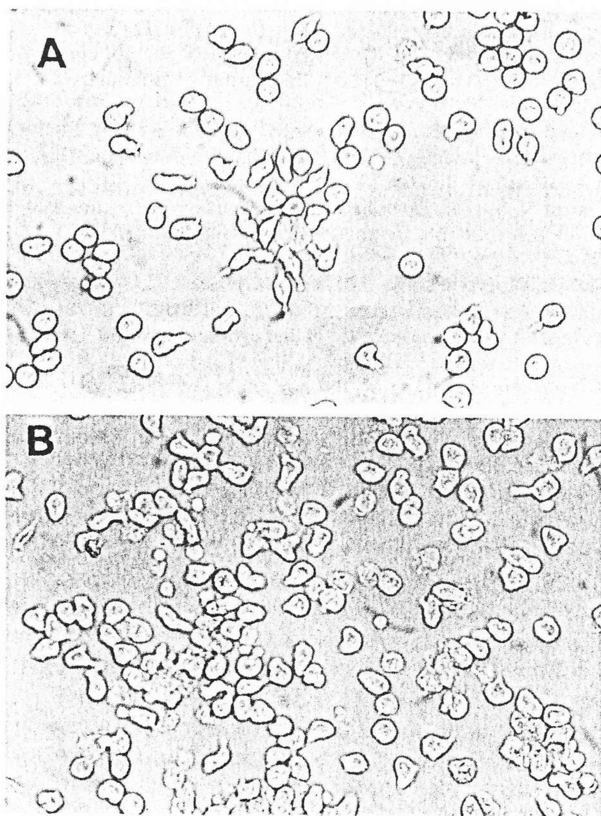

Fig. 2. Adherent PMN both resting (**A**) and Con A activated (**B**). A percentage of cells have elongated cytoplasm and are "tadpole-like" in appearance. PMN shape changes are less frequent in the middle of the Con A-activated cells

treatment. A proportion of Con A-activated PMN had an elongated cytoplasm forming a squat pseudopod. A higher percentage of these "tadpole-like" cells showed through the clear sides of the cuvettes with adhesion of resting PMN.

Discussion

In this study we have assessed PMN adhesiveness using FBS-coated polypropylene spectrophotometric cuvettes. The procedure involves the rotation at 90° of cuvettes resting on their major axis, at fixed times. Serum-coated, as opposed to uncoated, cuvettes were used for three reasons: (1) to standardize the procedure and allow for the different composition of the plastic walls of the cuvettes; (2) to assure uniform distribution of liquid along the inner walls (water repellent surfaces of uncoated cuvettes interfere with the damping of the whole surface); (3) to minimize PMN activation by uncoated plastic surfaces.

Rotation allowed contact of the highest number of cells with a large surface area. Finally, spectrophotometric cuvettes were used since they are of standard size, are readily available commercially and because they permit the microscopic examination of the cells adherent to the clear sides.

Two incubation times were selected. After a 16-min incubation, virtually all Con A-activated PMN adhered to the cuvette and there was a mean difference of 68% with the percentage adhesion of resting PMN. An 8-min incubation yielded approximately 50% adhesion of Con A-activated PMN and a mean 29%–30% difference compared with resting PMN.

The use of a 16-min incubation may allow the identification of severe defects of adhesion, whereas a shorter 8-min incubation may also be used to study in vitro the effects of drugs or substances which may enhance or decrease the intrinsic PMN adhesiveness.

As far as the clinical applications of our assay are concerned, it should be pointed out that in this study we have only investigated basal and Con A-stimulated adhesion. Here we used Con A because previous experience showed that the adhesion mediated by this lectin is higher than with other agonists like N-formyl-methiomyl-leucinyl-phenyl-alanine (fMLP), serum-treated zymosan and phorbol myristate acetate (PMA) [3]. Using the same procedure and assay medium, the adhesion stimulated by PMA (up to 1 μg/ml) was very low (data not shown). Moreover, the mechanisms of neutrophil adhesion are very complex and consequently various clinical defects are possible, depending on the lack of either integrins [2, 22, 23] or ligands for selections [6]. Since it is known that Con A-mediated adhesion involves both integrin-dependent and integrin-independent mechanisms [3, 19, 20], our test may be suitable for screening a wide range of defects. However, the use of this test in the identification of neutrophil defects related to the determinants of Con A-mediated adhesion requires investigation.

Microscopic examination of adherent cells through the clear walls of cuvettes allows a semi-quantitative evaluation of adhesion, thus confirming the marked activation due to Con A treatment (Fig. 2). In addition, selected shape changes associated with adhesion and activation may be observed. Adherent cells exhibited pseudopod formation accompanied by bipolar shape configuration in 23%–26% of resting cells and 15%–18% of Con A-activated cells. The presence of a small subpopulation of

elongated bipolar cells in resting neutrophils that were left to adhere to serum-coated glass was previously observed [18]. The decrease in the percentage of bipolar cells after treatment with Con A has not been previously reported; the most likely explanation of this finding is the rapidity and intensity of interaction between activated cells and adhesion substrate. As a consequence of such a strong interaction, the membrane of activated neutrophils spreads on the adhesion surface in multiple directions before the cells may take on a bipolar shape change. It is well known that Con A induces rapid (less than 2 min) PMN microtubule assembly with active pseudopod formation [11].

In conclusion, the suggested procedure offers several technical advantages over other adhesion assays already reported [10, 15], since it is simple, rapid and precise. The usefulness of this test in the study of human neutrophil pathology is under investigation.

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