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Assessment of neutrophil aggregation by Coulter® STKR and STKS haematological analysers

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Summary We have studied an alternative method to aggregometry for the assessment of human polymorphonuclear (PMN) leucocyte aggregation. This simple, rapid and reliable procedure counts unaggregated cells on both Coulter^R STKS and STKR haematological analysers by the impedance principle. Aggregation of PMN was induced by 15 min incubation with fresh autologous serum (FAS) after a 10 min phorbol myristate acetate (PMA) activation of neutrophils in small aliquots (0.25 ml) of suspension containing about 4.0×10^9 PMN/l. Differences ($\times 100$) between count of resting and PMA + FAS treated neutrophils/count of resting PMN reflect percent aggregation. By this procedure, PMN aggregation did not occur in autologous plasma from EDTA anticoagulated whole blood; it was partially inhibited by hydrocortisone, whereas inactivated or Zymosan activated sera gave values similar to those from FAS induced aggregation. PMA aggregation was dependent on $Ca^{2+} + Mg^{2+}$ concentration. Intra-assay analytical variability did not exceed 4% on either instrument. Reference values ($n = 20$) of percent PMN aggregation were 50.7 ± 4.7 on STKS and 47.1 ± 4.8 on STKR. Most probably, the interindividual variance was due to the physiological variability of Mg^{2+} and/or Ca^{2+} concentrations in FAS. Thus, this procedure reflects the true PMN aggregability status in a given subject, and in a given electrolyte environment.

Keywords: neutrophil aggregation *in vitro*, Coulter^R STKR, STKS analysers

Increasing interest in polymorphonuclear (PMN) leucocyte aggregation in biology and medicine arises from its pathogenetic role in many diseases (Craddock *et al.* 1979; Jacob *et al.* 1980). PMN aggregation is involved in the generation of vascular damage during various inflammatory conditions such as sepsis (Jacob 1980) and pneumococcal bacteraemia (Reed *et al.* 1984), but also occurs in patients undergoing extracorporeal circulation (Craddock *et al.* 1977a), after surgery (Elliott *et al.* 1985), shock, myocardial infarction (Jacob 1983) or granulocyte transfusion (Campana *et al.* 1985). PMN aggregation and pulmonary leucostasis may promote severe pulmonary damage as occurs in adult respiratory

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distress syndrome (Rinaldo & Rogers 1982). Cytochalasin B, N-formyl-methionyl-leucyl-phenylalanine (FMLP), phorbol myristate acetate (PMA), calcium ionophore A23187, arachidonic acid and leucotrienes elicit PMA aggregation and are the most frequently used stimuli for its study *in vitro* (Craddock *et al.* 1977b; O'Flaherty *et al.* 1978, 1980; Ringertz *et al.* 1982, 1985). Fresh plasma and, better still, Zymosan-activated plasma or serum produce the same effect (Craddock *et al.* 1977a; Abramson *et al.* 1983; Berliner *et al.* 1987). Aggregation induced by the former group of stimuli measures the intrinsic aggregation capacity of neutrophils (Wallis *et al.* 1986), whereas PMN aggregation by plasma or serum, both unactivated and activated, also reflects humoral effects on the aggregation (Craddock *et al.* 1977c; Hashimoto & Hurd 1981; Camussi *et al.* 1981; Abramson *et al.* 1983; Ringertz *et al.* 1985; Berliner *et al.* 1987). On the contrary, high-dose corticosteroids inhibit PMN aggregation *in vivo* and *in vitro* (Schumer 1976; Hammerschmidt *et al.* 1979; Skubitz *et al.* 1981).

Aggregometry by standard platelet aggregometer/recorder is the most widely employed method to study PMN aggregation (Craddock *et al.* 1977c; Craddock *et al.* 1977b; Oseas *et al.* 1980; Metcalf *et al.* 1986). Aggregation assay of PMN on whole blood is affected by concomitant aggregation of platelets and mononuclear leucocytes and therefore does not reflect the specific PMN response to aggregants. Aggregometry on isolated neutrophils has two main disadvantages: (1) the results do not really reflect the clinical condition of the patient (Weinberg *et al.* 1984), so this approach has been generally used as an indirect assay of the aggregation potential of the plasma, and not the aggregation state of the patient's neutrophils (Berlinter *et al.* 1987); (2) differences in light transmission may be induced not only by PMN aggregation but also by physico-chemical changes of cellular behaviour in response to many aggregants. These occurrences have been clearly demonstrated by PMN degranulation and polarization following stimulation with FMLP (Zigmond 1977; Kawaoka *et al.* 1981; Yuli & Snyderman 1984), by degranulation and swelling of neutrophils activated with PMA (Grinstein *et al.* 1986; Lippi *et al.* 1992a, 1992b) or other shape changes (Metcalf *et al.* 1986).

Neutrophil aggregation may be also evaluated on whole blood by light microscopy of stained smears according to Fleck's method (Fleck 1947) recently modified to a simpler test (Berliner *et al.* 1986, 1987). This procedure is time-consuming and affected by inter- and intra-observer differences in aggregated and unaggregated cell counts.

This paper describes a simple, rapid and reliable method for assaying PMN aggregation *in vitro* on two Coulter^{R} (STKR and STKS) haematological analysers. Both the STKR and STKS perform particle counting and sizing by the impedance method (Jones 1982). Unlike the platelet count for aggregation assay by the electrical impedance system (Sweeney *et al.* 1989) the leucocyte count on the STKR and STKS does not exclude microaggregates by a fixed volumetric threshold, thus unaggregated cells and microaggregates are enumerated together. On the contrary, macroaggregates that do not pass through the aperature (100 μm diameter) of the counting systems do not interfere with cell count. Since, in a preliminary study, we observed negligible microaggregates compared with

unaggregated cells, the difference ($\times 100$) between counts from cell suspensions before and after induced PMN aggregation divided by count from untreated suspensions was considered a measurement of percent aggregation.

In this study neutrophil aggregation is induced on separated PMN by adding fresh autologous serum to PMN suspensions in phosphate-buffered saline solution (PBS), previously treated with PMA.

Materials and methods

REAGENTS

Ficoll (Type 400) d 1077 and 1119, containing sodium diatrizoate (Histopaque 1077 and 1119), dimethyl sulphoxide (DMSO), PMA, Zymosan A and PBS were purchased from Sigma Chemical Co., St. Louis, MO, USA. Disodium succinate hydrocortisone (HC) (Solu-Cortef 125 mg/l, Upjohn, Puurs-Belgium), was diluted with PBS in stock solutions containing HC 2.2, 5.5 and 11 mg/ml. PMA was dissolved in DMSO at a concentration of 5 mg/ml and stored in aliquots at $-20°C$. Immediately before use, the PMA solution was diluted in PBS at a concentration of 10^{-3} mg/ml. Zymosan was suspended at 20 mg/ml in NaCl 0.15 mol/l, boiled for 30 min, washed twice, and resuspended in NaCl 0.15 mol/l at a final concentration of 20 mg/ml; aliquots of 5 μl were stored in small test tubes at $-20°C$. Serum complement activation was produced in fresh autologous serum (FAS) by incubating 0.5 ml aliquots of FAS with 5 μl of Zymosan suspension (0.2 mg/ml of FAS), with tumbling, at 37°C for 30 min (Hammerschmidt *et al.* 1980). The Zymosan activated autologous serum (ZAAS) was then cooled rapidly to 4°C, centrifuged 14 000 min^{-1} for 2 min in an Eppendorf Centrifuge 5415, and decanted from the Zymosan button.

METHODS

The subjects were 20 apparently healthy volunteers, mean age 36.8, SD 11.4 (8 males and 12 females), range 19–58 years, who gave informed consent. Subjects were non-smokers and had not undergone any pharmacological treatment during the previous two months. Venous blood was collected in polypropylene tubes containing K_3 EDTA (1.5 mg/ml). PMN were isolated on double discontinuous density gradient (Histopaque 1077–1119) according to the Sigma Revised Procedure No 1119. Red blood cell (RBC) contamination was excluded in some suspensions by hypotonic lysis, treating PMN pellets with cold distilled water. PMN pellets were gently resuspended in small volumes of PBS and suspensions were filtered through Seraclear (Bayer Diagnostics, Milan, Italy).The device was thrust 3–4 cm into a polypropylene test tube (1.5×10 cm). Suspensions were poured into the Seraclear and filtered by slowly removing the device from the tube. By this means, fragments of unbroken pellet were eliminated and instrument blockage during cell counting avoided. PMN suspensions were then diluted with

PBS to fixed cell concentrations of 6.7, 9.0 and 11.3×10^9/l. Final PMN concentrations in the aggregation tests approximated 3.0, 4.0 and 5.0×10^9/l, respectively. Cell viability of separated PMN was assessed by the trypan blue exclusion test.

Aliquots (0.5 ml) of PMN suspensions were added to PBS (0.4 ml) in siliconized glass tubes (5×0.7 cm) containing a Teflon stir bar revolving at 2000 rpm in a holder of a common aggregometer. After 5 min delay to allow warming of cells to 37°C, PMA solution (0.1 ml, final concentration: 100 ng/ml) and PBS (0.1 ml) were added to test and control tubes, respectively. After 10 min, one half of the suspension was taken off for PMN sizing on Coulter^R STKR and STKS (Coulter Corporation, Hialeah, FL, USA), and FAS or alternatively ZAAS (0.05 ml) was added to the remaining suspensions of PMA activated and resting neutrophils. Incubation (stir bar revolving at 1000 rpm) was carried on for 10, 15 and 20 min before cell counting on the two analysers. PMA aggregation was measured as:

$$\% \text{ aggregation} = \frac{\text{Resting count} - \text{PMA activated count}}{\text{Resting counting}} \times 100$$

At fixed times after adding serum to PMA activated and resting neutrophils, 2–5 μl of suspension were smeared on a slide and rapidly air dried. Smears were fixed with methanol and stained with May Gruenwald-Giemsa. Percentage of aggregated neutrophils were determined microscopically ($40 \times$) by counting 200 cells. PMN were considered aggregated if more than three were clumped. In preliminary experiments, fresh autologous plasma from EDTA anticoagulated whole blood or inactivated (30 min at 56°C) autologous serum were added, instead of FAS, to suspensions of PMA activated neutrophils.

The aggregating effect of bivalent cations ($Ca^{2+} + Mg^{2+}$) was also studied both at physiological level of final concentration or at a level resembling the cationic concentration in test tubes, after adding FAS. For this purpose we used alternatively a 0.15 mol/l NaCl stock solution containing Ca^{2+} 15.4 mmol/l and Mg^{2+} 7.7 mmol/l (chloride salts) and the same stock solution diluted 1 : 5, 1 : 10 and 1 : 20 with 0.15 mol/l NaCl. The effect of hydrocortisone (HC) was tested by measuring PMN aggregation in suspensions containing 0.2, 0.5, 1.0 mg/ml HC final concentrations. For this study, PBS (0.3 ml) and HC stock solution (0.1 ml) were added to PMN suspensions (0.5 ml). Prewarming, PMA activation and PMA + FAS-induced aggregation were performed as described before. PBS was added, instead of PMA, to the control tubes; after a 10 min incubation, FAS was also added.

Results

The PMN separation, by the one-step discontinuous gradient method, supplied yields of neutrophils between 25 and 47%. Purity ranged from 96 to 99.7% and viability of cells that excluded trypan blue always exceeded 95%. Addition of FAS to PMN suspensions showed aggregation of PMA activated neutrophils on both instruments by a decrease in cell counts when compared with those per-

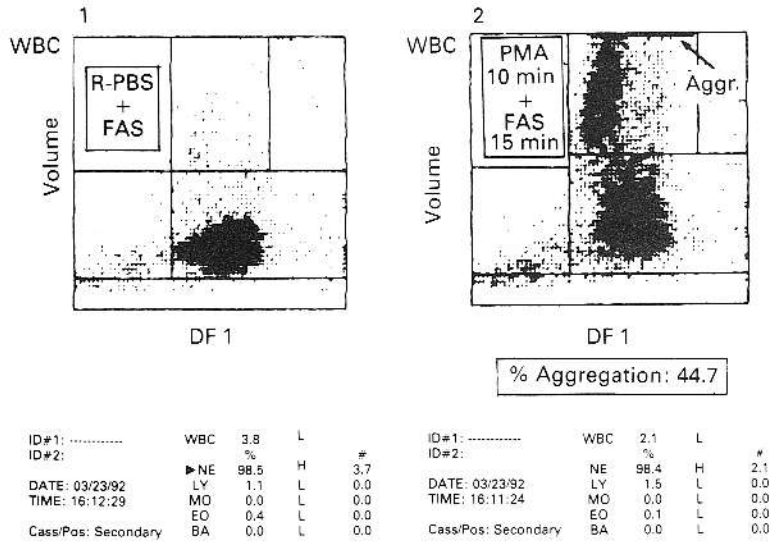

Figure 1. PMN purity (▶) in a representative PMN separation from EDTA anticoagulated whole blood (1.1). FAS-induced PMN aggregation (44.7%) was plainly found after a 15 min incubation of PMA activated neutrophils (1.2 *vs* 1.1).

formed on suspensions of resting FAS-treated neutrophils (Figure 1.2 *vs* 1.1 and Figures 2.4, 2.5, 2.6 *vs* 2.3). PMN aggregates could be plainly singled out on the DF1 display on the STKS, a composite scatterplot indicating the position of cells derived from aperture impedence volume and light scatter.

PMN aggregation was demonstrated on the STKR by the abnormal distribution of PMN volumes on leucocyte volume histogram with the presence of a cellular population larger than 300 fL (Figures 2.4, 2.5, 2.6 *vs* 2.3). If FAS was not added, PMN aggregation did not occur even after 25 min of PMA activation.

Added bivalent cations, at the physiological ionized level, induced gross aggregation of PMA activated neutrophils already after 10 min. Microscopic examination of smeared aggregated and unaggregated cells demonstrated, in $Ca^{2+} + Mg^{2+}$ treated PMN suspensions, voluminous aggregates including ten or more cells, which cannot pass through the instrument aperture. On the STKS scatterplot DF1, cells were scarce; cell counting was also flagged and was too low to be satisfactorily reliable.

The effect of bivalent cations at concentrations near to the concentrations present in control tubes, after adding FAS, is shown in a representative experiment reported in Figure 3. After 10 min PMA activation and a 15 min incubation with $Ca^{2+} + Mg^{2+}$ at three concentrations, PMN percent aggregation increased proportionately to the rise of Mg^{2+} content with a constant Ca^{2+}/Mg^{2+} ratio (= 2). FAS induced percent aggregation was a little higher than that obtained at Mg^{2+} 0.06 mmol/l (Ca^{2+} 0.12 mmol/l). Ionized Ca^{2+} and Mg^{2+} concentrations in the FAS were 1.54 and 0.79 mmol/l, respectively, with similar concentrations of bivalent cations in the two test tubes. Cellular distribution on the STKS volume-

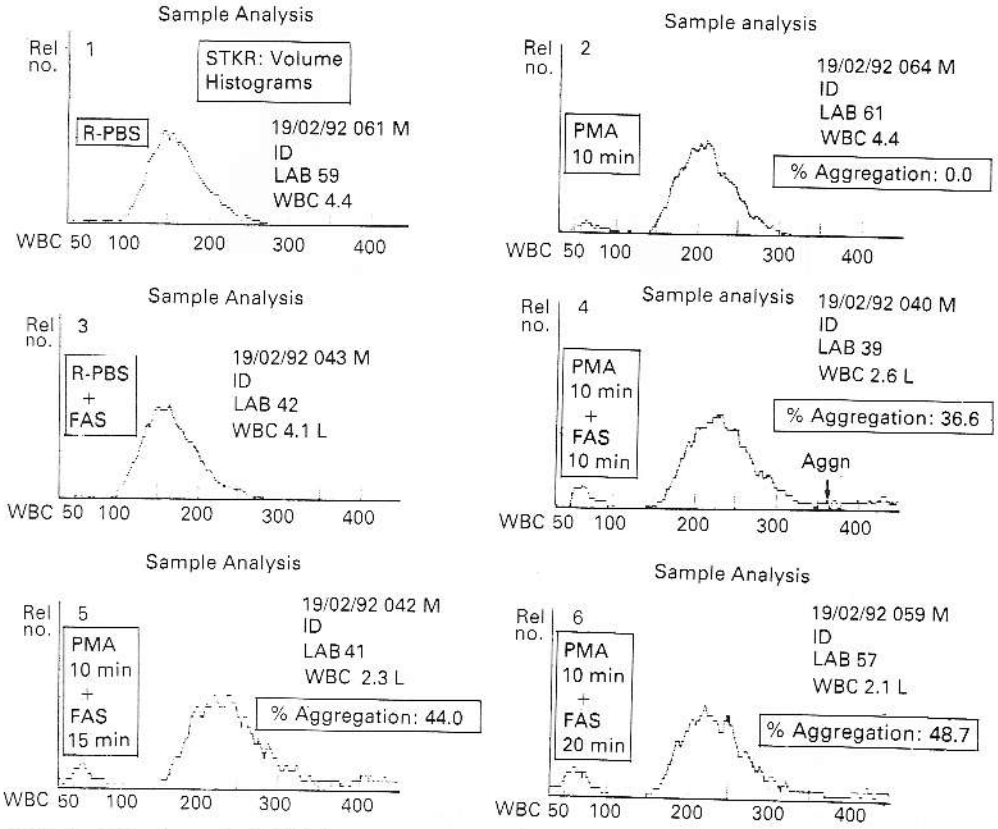

Figure 2. Time-dependent PMN percent aggregation after FAS incubation of PMA activated neutrophils. PMN aggregates showed as an abnormal distribution of volume on STKR leucocyte volume histograms with a cellular component larger than 300 fL (Aggr. in 2.4 *vs* Resting + FAS neutrophils in 2.3).

scatter DF1 scatterplot was different, lacking a population of unaggregated PMN with higher scatter in $Ca^{2+} + Mg^{2+}$ treated suspensions (Figures 3.4, 3.5 *vs* 3.2).

Autologous plasma from EDTA anticoagulated whole blood did not induce aggregation of PMA activated neutrophils, while ZAAS or inactivated autologous serum did not affect the magnitude of FAS induced aggregation in controls. PMN aggregation by FAS was either time-dependent (Figures 2, 4 and 5) or proportional to the cellular content of the suspensions (Figures 4 and 5). Suspensions containing approximately 4.0×10^9 PMN/l, activated 10 min with PMA and incubated 15 min after adding FAS, produced mean values of percent aggregation nearing 50% on both instruments.

Data regarding percent aggregation of PMN from 20 healthy subjects are reported in Table 1. There was no evidence of sex difference. The most satisfactory agreement for methods were between microscopy and STKS.

We studied the effect of RBC hypotonic lysis on PMA + FAS induced PMN aggregation. A 30 s RBC lysis by cold distilled water did not reduce the amount of percent PMN aggregation. Likewise, gross RBC contamination by adding autolo-

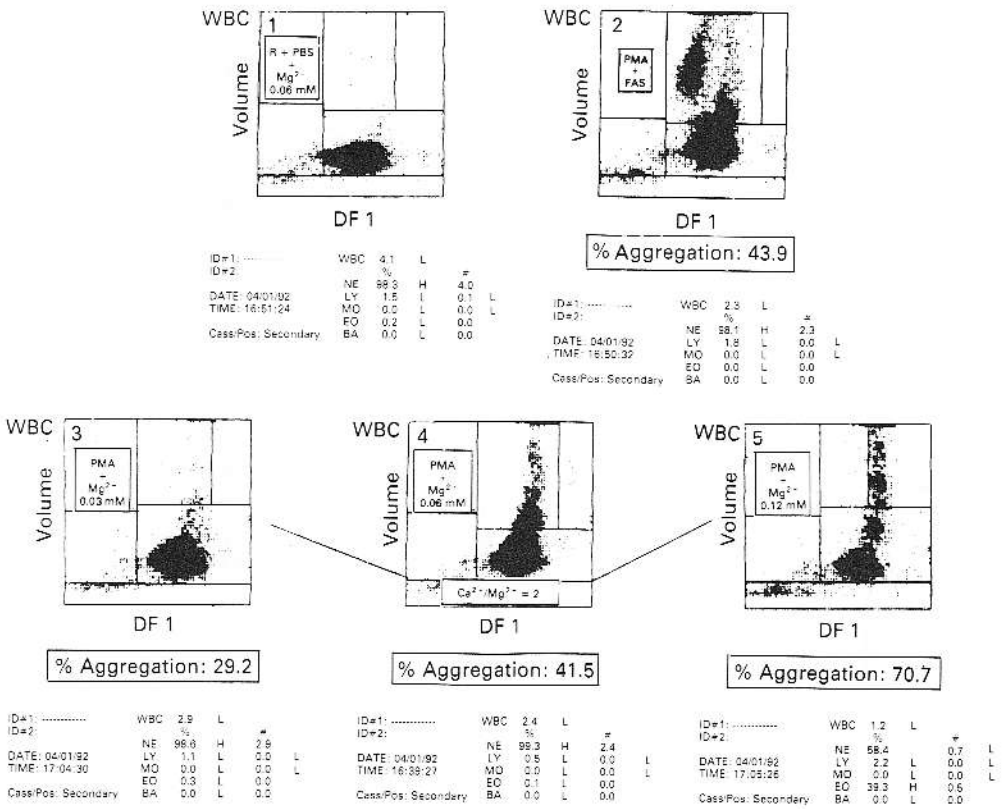

Figure 3. Instrumental data (STKS) in a representative experiment of PMA+FAS and PMA+(Ca^{2+} + Mg^{2+}) induced PMN aggregation, after 10 min PMA activation, showed that aggregation was proportional to the concentrations of bivalent cations in test tubes (3.3, 3.4 and 3.5). Resting PMN (R-PBS) (3.1) were incubated 10 min after adding Mg^{2+} and Ca^{2+}; PMN percent aggregation by PMA + FAS (3.2) was a little higher than that by Mg^{2+} 0.06 mmol/l (Ca^{2+}/Mg^{2+} = 2). Concentration of ionized Mg^{2+} in diluted FAS was 0.07 mmol/l. Since FAS and electrolyte-induced aggregation were stopped after 10 min incubation, percent aggregation values from these experiments were not taken into account to produce reference interval.

gous PBS-washed RBC to PMN suspensions did not interfere with PMN percent aggregation.

The effects of high-dose corticosteroids of PMN percent aggregation are also reported in Table 1. Dose-response of the inhibition, induced by HC to PMN aggregation, was not linear, as we noted large interindividual responsiveness to the same HC dose. In spite of this, decreased PMN aggregation was found at each final HC concentration.

Discussion

Our results suggest the following: (1) PMN aggregation of PMA activated neutrophils is elicited either by $Ca^{2+} + Mg^{2+}$ or FAS, (2). Percent aggregation is

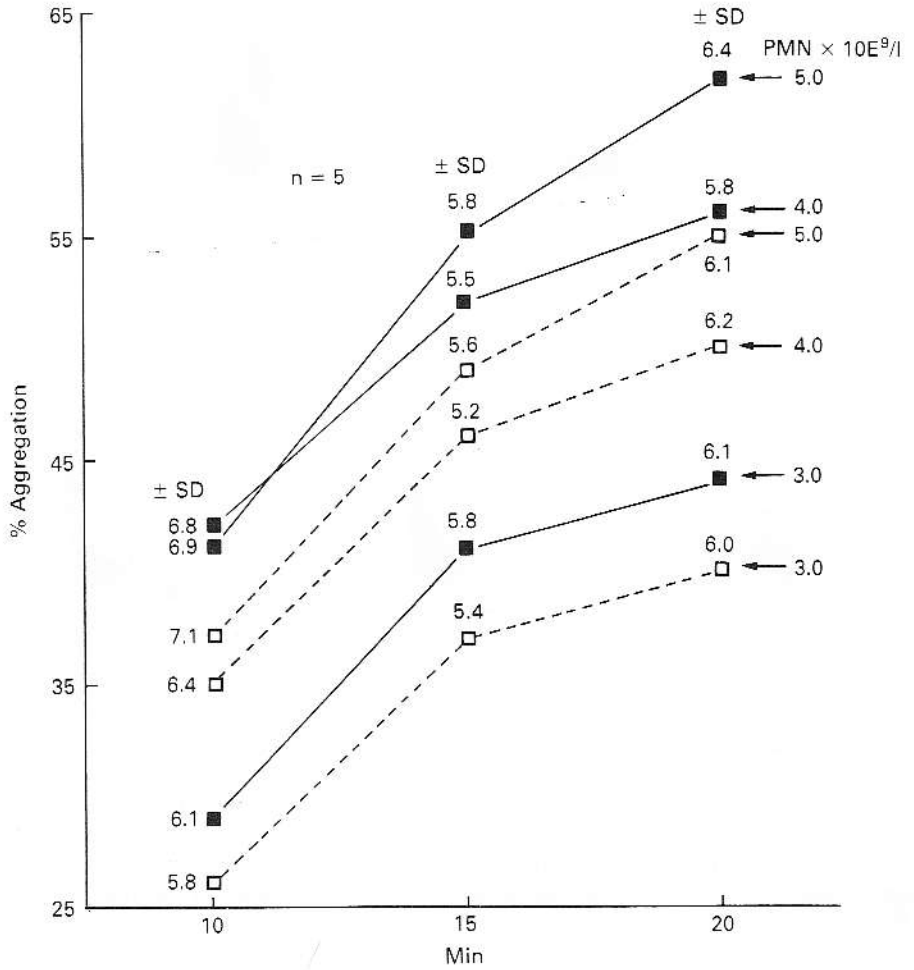

Figure 4. Mean (±SD) percent aggregation in five PMN suspensions containing different cellular concentrations at various times of FAS incubation. The entity of percent aggregation was time-dependent, in proportion with PMN contents of the suspensions and higher with STKS. —□— STKR; —■— STKS.

proportional to concentrations of bivalent cations in the test tubes, (3). Autologous plasma from EDTA treated whole blood does not induce PMN aggregation, whereas ZAAS and inactivated autologous serum does not affect percent aggregation values obtained with FAS.

Aggregation of PMA activated neutrophils requires bivalent cations ($Ca^{2+} + Mg^{2+}$) and Mg^{2+}, but not Ca^{2+}, can support PMA induced aggregation; Ca^{2+} increases the speed with which the phenomenon occurs (O'Flaherty *et al.* 1980).

At a final concentration of bivalent cations similar to that present in test tubes, after adding FAS, percent PMN aggregation was almost of the same magnitude. Since there is physiological variation in Mg^{2+} and Ca^{2+} concentrations in FAS

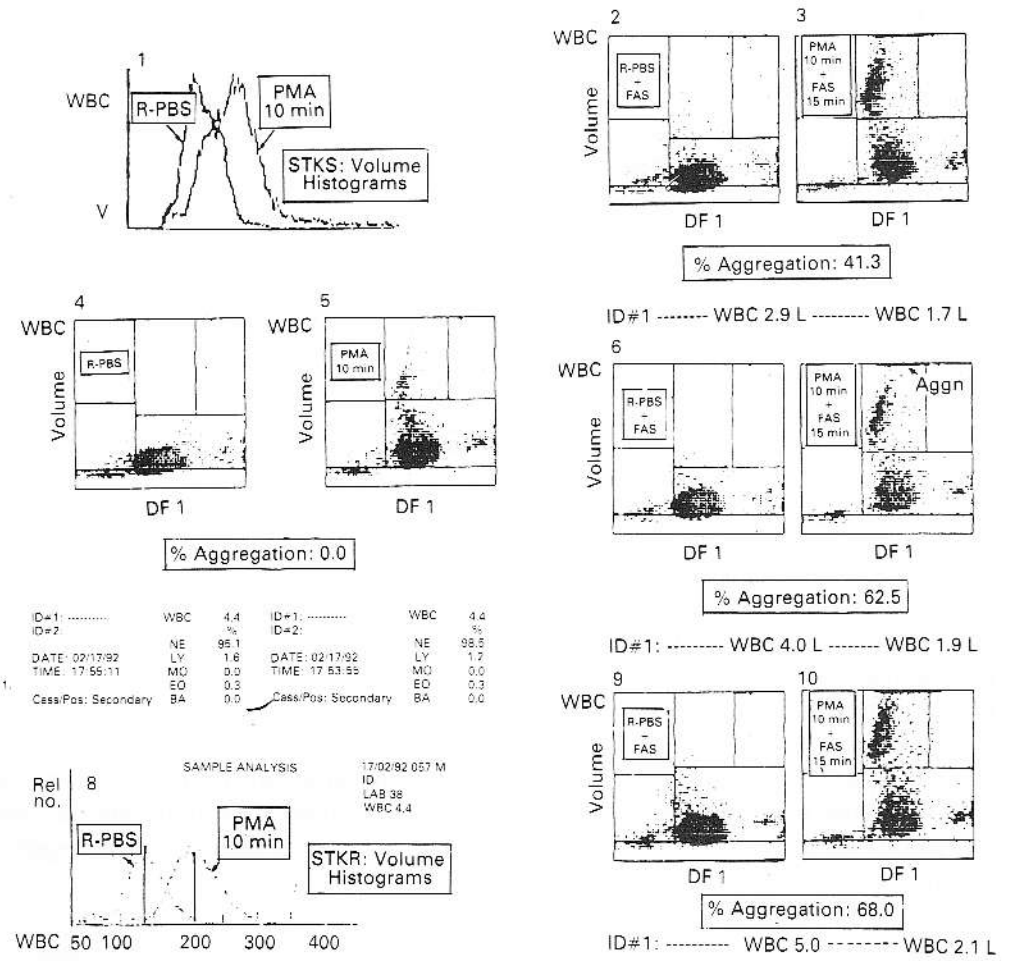

Figure 5. A representative case of PMN volume changes assayed on STKS (5.1) and STKR (5.8) after PMA activation. FAS induced aggregation of PMA activated neutrophils was in proportion with the cellular content of FAS treated resting neutrophils (R-PBS+FAS) in the suspensions. Different PMN concentrations (2.9, 4.0 and 5.0×10^9/l) were used with increased percent aggregation from 41.3 to 58% (5.3, 5.7 and 5.10).

from different subjects, we preferred to study the PMN aggregation by FAS as representative PMN aggregability in a given subject, in a given plasmatic environment.

PMN percent aggregation, assayed *in vitro* by this procedure, depended on PMN concentrations and FAS incubation times, after PMA activation. Since RBC contamination did not interfere with the FAS induced aggregation, hypotonic lysis of RBC was not taken into account.

Cellular concentrations were such that the smallest PMN amount could be used with a number of unaggregated cells within the range of instrumental linearity, precision and accuracy of counting. Counts lower than 1.5×10^9/l are often flagged and are less reliable. PMN final concentration of 4.0×10^9/l, 10 min

Table 1. Evaluation of percent neutrophil aggregation by STKS or STKR and microscopy in 20 PMN suspensions of healthy non-smoker subjects and percent inhibition of aggregation by hydrocortisone (HC) ($n = 6$)

Methods	Percent aggregation $\bar{X} \pm SD$ (ranges)	Percent inhibition of aggregation by HC (mg/ml) $\bar{X} \pm SD$ (ranges)		
		0.2	0.5	1.0
Microscopy	$50.4 \pm 4.6^{a*}$ (43–58)			
STKS	$50.7 \pm 4.7^{b*}$ (43–59.5)	11.8 ± 7.9 (5–22)	17.0 ± 7.8 (9–27)	19.5 ± 8.2 (10–30)
STKR	$47.1 \pm 4.8^{c*}$ (37.8–55.6)	11.7 ± 8.8 (4–24)	17.8 ± 8.0 (9–28)	18.5 ± 8.3 (9–29)

*Significance by Students' paired *t*-test: a *vs* b, $P > 0.1$; a *vs* c and b *vs* c, $P < 0.001$.

of PMA activation and a 15 min FAS incubation gave mean values nearing 50%, allowing assessment of even wider variations of enhanced or decreased percent aggregation. When aliquots of the same suspension of resting PMN were retested for aggregation, differences did not exceed 4% on both analysers. Counts of unaggregated cells carried out within 15 and 17 min averaged variations of less than 2% at PMN concentration of 4×10^9/l (Figure 4). The higher values of percent aggregation obtained on STKS, as compared with those assayed on STKR (Table 1) cannot be accounted for, even though both analysers perform cell counts by the same impedance system. The agreement of percent aggregation by microscopy with STKR or STKS demonstrated that the number of aggregates counted along with the unaggregated cells was negligible.

Our results confirmed the inhibitory role of corticosteroids, at high dosage, on PMN aggregation (Skubitz *et al.* 1981). To explain this, an effect that excludes the FAS action on aggregation should be invoked. Corticosteroids block the release of lactoferrin to a much greater extent than the lysozyme from neutrophils on stimulation with PMA (Oseas *et al.* 1982). Since lactoferrin can enhance PMN adherence to endothelial cells and promote aggregation of neutrophils as well, it seems reasonable that, following cell activation, corticosteroids might selectively impair the release of specific granule contents that contribute to leucocyte stickiness (Oseas *et al.* 1981).

The study confirmed the remarkable interindividual variance of PMN percent aggregation *in vitro* (Berliner *et al.* 1987) which we believe is due, in our experimental model, to the wide range of Mg^{2+} (and perhaps Ca^{2+}) concentrations in serum of healthy subjects. The reference interval for PMN percent aggregation is perhaps too wide for the estimation of *in vitro* PMN hyperaggregability. In spite of this fact, the practicability and the precision of the procedure allows the study of *in vitro* PMN aggregation response to both activators or inhibitors, and appears very suitable for pharmacological research. The effects of activators or inhibitors of PMA aggregation can be evaluated in a given subject using FAS, or

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alternatively employing a fixed concentration of aggregating cations ($Mg^{2+} + Ca^{2+}$) to study PMN intrinsic aggregability in a more standardized manner.

We therefore recommend this quick and reliable procedure for *in vitro* assay of human PMN aggregability on two analysers commonly found in haematology laboratories.

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