Chapter 4

Leukocyte cell population data (VCS) in postprandial leukocyte activation

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Abstract

Introduction: Changes in leukocyte cell population data have been reported in various infectious diseases, but little is known in other inflammatory conditions such as the postprandial state. We investigated whether leukocyte cell population data change during postprandial leukocyte activation.

Methods: Healthy volunteers underwent a standardized oral fat loading test (OFLT). Flowcytometric quantification of leukocyte activation markers CD11b, CD66b, CD35 and CD36, together with leukocyte cell population data from LH750 hematology analyzers were measured fasting and at 4 and 8 hours postprandially.

Results: Twelve volunteers were included. Postprandial leukocyte activation was confirmed by increased expression of CD11b by monocytes (+11.7%) and neutrophils (+15.0%) and by increased expression of CD66b (+14.7%) and CD35 (+16.6%) by neutrophils at T = 4h. The mean scatter from neutrophils, reflecting granularity, significantly decreased at T = 4h ($P < 0.05$) and returned to baseline at T = 8h (P-ANOVA = 0.048). The mean volume of monocytes increased significantly at T = 4h ($P < 0.001$) and returned to baseline at T = 8h (P-ANOVA = 0.0008). At T = 4h CD11b expression on neutrophils was associated with a reduction in mean scatter of neutrophils (Pearson's r: -0.677, P = 0.016).

Conclusion: Postprandial leukocyte activation is accompanied by temporary changes in leukocyte cell population data, similar to changes observed during various infections, but to a lesser extent.

Introduction

Triglyceride-rich lipoproteins are abundant in the postprandial phase and are able to induce an inflammatory response, which includes postprandial leukocyte activation [1]. Neutrophils are recruited within two hours after the ingestion of fat and/or glucose together with concomitant impairment of vessel dilatation [2,3]. In vitro experiments have shown that chylomicron remnants activate monocytes and neutrophils [4,5]. Moreover, postprandial leukocyte activation is observed in vivo after the ingestion of a fat-containing meal, which directly correlates with the postprandial increase in triglycerides (TG) [5-7]. Postprandial activated monocytes can adhere on endothelial cells by increased expression of adhesion molecules, mainly CD11c/CD18 [7], which may facilitate subendothelial migration of monocytes, with risk of foam cell formation and subsequently the development of atherosclerosis [1].

Changes in leukocyte cell population data have been reported in various infectious diseases. Monocyte and neutrophil volume temporarily increases combined with decreased light scatter by neutrophils during bacterial infections or sepsis [8-10]. In contrast, little is known about changes in leukocyte cell population data during other non-infectious inflammatory conditions like the inflammatory postprandial state. Leukocyte cell population data are acquired during the 5-part leukocyte differentiation by automatic cell counters in clinical laboratories. The LH750 hematology analyzer (Beckman Coulter, Miami, USA) uses Volume Conductivity Scatter (VCS) technology to perform the 5-part leukocyte differential count. Different parameters of VCS-data reflect the granularity and activation state of leukocytes. Over 8000 cells are measured using direct current impedance to measure cell volume (V). A radio frequency probe measures conductivity (C) that reflects nuclear shape, lobularity and nuclear/cytoplasmic ratio. Finally, laser light scatter (S) quantifies cellular granularity [11,12]. Together, these leukocyte cell population data are used to identify the different leukocytes for the 5-part differential count. Recently, it has been suggested that leukocyte cell population data may also be a clinical useful indicator for acute bacterial infections [8,10], postoperative infections [13], malaria [14] and as a discriminator between fungemia and bacteremia [15].

Since postprandial lipemia is one of the most physiological stimuli for leukocyte activation, our aim was to study whether changes in leukocyte cell population data occur postprandially and whether there is a concomitant relationship with leukocyte activation markers.

Materials and methods

Subjects and study design

Male and female volunteers were recruited by advertisement. Subjects had to be healthy and aged between 18 and 50 years. Exclusion criteria were the use of any medication or the presence of hypertriglyceridemia (fasting TG > 2.00 mmol/l). Participants visited the hospital after a 10h overnight fast. Anthropometric characteristics like length, weight, body mass index, waist circumference and blood pressure were measured. A fasting venous blood sample was drawn (T = 0h) and subjects were given an oral fat load using fresh cream (Fresh cream, Albert Heijn, Zaandam, the Netherlands) in a dosage of 50g of fat per square meter body surface at 9:00 AM. During the oral fat loading test (OFLT) participants were not allowed to smoke, eat or drink (except water) and participants refrained from physical activity. Venous blood samples were taken at 4 (T = 4h) and 8 hours (T = 8h) after the ingestion of the oral fat load. The time-points T = 4h and T = 8h were chosen since postprandial triglyceridemia in healthy subjects is maximal at T = 4h and TG return to fasting levels at T = 8h. In addition, it has been shown that postprandial leukocyte activation is highest at T = 4h [5]. The study was approved by the independent Regional Medical Ethical Committee Rotterdam (Maasstad Hospital, the Netherlands) and all subjects gave written informed consent.

Clinical chemistry

All clinical chemistry measurements were carried out on freshly drawn blood in the department of Clinical Chemistry, Sint Franciscus Gasthuis, Rotterdam. Baseline renal function, C-reactive protein, glucose, plasma cholesterol, HDL-C and triglycerides were measured using the LX20 or DxC analyzers (Beckman Coulter, Miami, USA). LDL-C values were calculated using the Friedewald formula. Apolipoprotein A-I and apo B were determined by nephelometry using an IMAGE instrument with commercially available kits (Beckman Coulter).

Leukocyte VCS-data

Blood cell counts and 5-part leukocyte differentiation were determined automatically using LH750 analyzers (Beckman Coulter) within 45 minutes after venipuncture. VCS-data were obtained from lymphocytes, monocytes and neutrophils. These data were generated by optical and electronic measurements of individual cells by the LH750 analyzers during generation of the automated leukocyte 5-part differential count. The parameters include the mean channel and standard deviation of volume (V), conductivity (C) and light scatter (S) for lymphocytes, monocytes and neutrophils. Using a normal blood sample that was measured 10 consecutive times on the LH750 we established the coefficient of variation (CV) for each of these parameters. With the exception of lympho-

cyte conductivity (CV: 1.41%) all CV's were below 1%. This indicates that VCS-data are robust and have very little analytical variation, as was also reported by others [10].

CD11b, CD66b, CD35 and CD36 as markers for *in vivo* leukocyte activation

Blood samples for the determination of leukocyte activation markers CD11b, CD66b, CD35 and CD36 were collected in tubes containing 5.4mg K2 EDTA (Becton Dickinson, Plymouth, United Kingdoms) at baseline and at four and eight hours after ingestion of the oral fat load. The method has been described in detail before [5,6]. Briefly, the staining procedure was started within 15 minutes after venipuncture. All measurements were carried out in triplicate. Separate tubes were prepared: 1) a combination of fluorescein isothiocynate (FITC) conjugated CD66b, phycoerythrin (PE) conjugated CD11b and phycoerythrin-Texas Red-X (ECD) conjugated CD45 and 2) a combination of FITC conjugated CD36, PE conjugated CD35 and ECD conjugated CD45. All antibodies were from Beckman Coulter, except for CD35-PE (BD Biosciences, Franklin Lakes, NJ, USA). A total of 20 µl of whole blood was added to each tube and incubated for 15 minutes in the dark on room temperature. Erythrocytes were lysed by addition of 500µl lysis solution (1.5 M ammonium chloride, 100 mM potassium hydrogen carbonate, 0.82 mM EDTA, pH7.4) followed by 15 min of incubation in the dark. The samples were measured on a Navios flow cytometer (Beckman Coulter). Samples were measured for a maximum of five minutes or until at least 2000 monocytes were acquired. Lymphocytes, monocytes and neutrophils were identified in the side scatter versus CD45 dot plot. The fluorescence intensity of each cell type was expressed as the mean fluorescence intensity (MFI) of the triplicate measurements. Before each use, the optics and settings of the flow cytometer were checked with Flow-Check Pro and Flow-Set Pro beads (Beckman Coulter). Identi-

Table 1: Antibody information concerning the measurements of *in vivo* leukocyte activation by flow cytometry.

FL-channel	Marker	Intra-assay variation (%, range)		Dilution	Antibody clone	Catalog number	Company
		Monocytes	Neutrophils				
FITC	CD66b	NA	1.8% (0.4-9.5)	20x	80H3	0531	BC
FITC	CD36	5.6% (1.0-17.0)	NA	5x	FA6.152	PN IM07664	BC
PE	CD35	4.4% (0.6-11.1)	4.0% (1.9-7.0)	1x	E11	559872	BD
PE	CD11b	2.5% (0.2-6.3)	2.5% (0.8-6.3)	10x	Bear1	PN IM2581	BC
ECD	CD45	NA	NA	5x	J33	A07784	BC

The intra-assay variation is shown in relative terms (%) as the coefficient of variation of the triplicate measurements of the respective antibody and cell type in the fasting state. The mean and range of all subjects is given.

Abbreviations: fluorescein isothiocynate (FITC), phycoerythrin (PE), phycoerythrin-Texas Red-X (ECD), not applicable (NA), Beckman Coulter (BC), Becton Dickinson Biosciences (BD).

cal instrument settings were used for the complete study. An overview of the antibody information and the intra-assay variation of the respective *in vivo* leukocyte activation markers is given in Table 1.

Statistical analysis

Data are given as mean ± SD in the text and tables and as mean ± SEM in the figures unless stated otherwise. The relative intra-assay variation in markers for *in vivo* leukocyte activation by flow cytometry was assessed by calculating the coefficient of variation (CV) of the triplicate measurements in the fasting state of the respective marker for each subject. The CV was calculated for each specific marker and cell type as: (standard deviation / mean MFI) * 100. Postprandial time effects were analyzed using repeated measures ANOVA with Dunnett's Multiple Comparison Test as post hoc analysis. VCS-data of one time-point (t = 8h) was missing in two volunteers and these data were imputed by using the mean of the respective individual. Correlations were calculated using the Pearson's correlation coefficient. PRISM version 5.0 (Graph Pad Software, San Diego, USA) and PASW 18.0 (IBM, New York, USA) were used for statistical analyses. Statistical significance was set at $P < 0.05$ (two-sided).

Table 2: Characteristics of the subjects. Data are given as mean ± standard deviation, unless stated otherwise.

	n = 12
Age (years)	35.3 ± 12.7
Male gender (n, %)	6 (50)
Body mass index (kg/m^2)	21.9 ± 2.1
Systolic blood pressure (mmHg)	110.5 ± 9.1
Diastolic blood pressure (mmHg)	65.1 ± 8.8
Leucocyte count (*10^9/l)	6.1 ± 1.1
Absolute lymphocyte count (*10^9/l)	1.7 ± 0.3
Absolute monocyte count (*10^9/l)	0.46 ± 0.13
Absolute neutrophil count (*10^9/l)	3.8 ± 0.9
C-reactive protein (mg/l)	1.4 ± 0.7
Total cholesterol (mmol/l)	4.6 ± 0.6
LDL-C (mmol/l)	2.7 ± 0.6
HDL-C (mmol/l)	1.63 ± 0.39
Triglycerides (mmol/l)	0.71 ± 0.24
Apolipoprotein B (g/l)	0.78 ± 0.14
Apolipoprotein A-I (g/l)	1.88 ± 0.35

Table 3: Postprandial changes in triglycerides and absolute counts for total leukocytes, lymphocytes, monocytes and neutrophils after a standardized oral fat load (n = 12). Data are given as mean ± standard deviation.

	T = 0h	T = 4h	T = 8h	P-ANOVA
Triglycerides (mmol/l)	0.71 ± 0.24	1.62 ± 0.70***	0.81 ± 0.57	<0.001
Total leucocyte count (*10^9/l)	6.1 ± 1.1	6.7 ± 1.5	7.5 ± 2.0***	0.0017
Absolute lymphocyte count (*10^9/l)	1.8 ± 0.3	2.0 ± 0.4*	2.4 ± 0.6***	<0.001
Absolute monocyte count (*10^9/l)	0.46 ± 0.13	0.45 ± 0.14	0.54 ± 0.15***	0.002
Absolute neutrophil count (*10^9/l)	3.8 ± 0.9	4.3 ± 1.4	4.4 ± 1.9	0.26
Relative lymphocyte count (%)	28.3 ± 4.8	29.5 ± 6.5	33.1 ± 8.6*	0.04
Relative monocyte count (%)	7.6 ± 1.9	7.0 ± 1.8	7.3 ± 1.8	0.17
Relative neutrophil count (%)	62.5 ± 6.3	61.8 ± 8.3	57.6 ± 10.4	0.06

* Significantly different from corresponding value at T = 0h (P < 0.05)
*** Significantly different from corresponding value at T = 0h (P < 0.001)

Table 4: Postprandial changes in VCS-data after a standardized oral fat loading test (n = 12). Data represent the mean ± standard deviation.

	T = 0h	T = 4h	T = 8h	P-ANOVA
Lymphocytes				
V-MN	81.2 ± 2.7	81.5 ± 2.3	80.5 ± 2.5	0.35
V-SD	13.7 ± 0.8	13.5 ± 1.2	13.2 ± 0.8	0.24
C-MN	114.0 ± 1.6	114.1 ± 1.9	116.0 ± 2.3*	0.009
C-SD	10.5 ± 1.6	9.8 ± 1.1	9.9 ± 1.2	0.22
S-MN	67.3 ± 3.1	67.7 ± 3.2	67.6 ± 4.3	0.87
S-SD	16.3 ± 1.4	16.1 ± 1.8	15.9 ± 1.7	0.69
Monocytes				
V-MN	162.4 ± 4.3	165.0 ± 4.8***	163.5 ± 4.2	0.0008
V-SD	17.2 ± 2.0	17.1 ± 1.6	17.3 ± 1.6	0.96
C-MN	123.4 ± 3.0	122.5 ± 3.2	124.8 ± 3.0	0.022
C-SD	4.5 ± 0.7	4.4 ± 0.4	4.5 ± 0.5	0.76
S-MN	90.0 ± 3.5	88.7 ± 2.7	89.5 ± 2.2	0.22
S-SD	9.6 ± 0.7	9.5 ± 0.7	9.4 ± 1.1	0.65
Neutrophils				
V-MN	140.8 ± 2.7	142.3 ± 3.8	140.9 ± 4.6	0.27
V-SD	18.4 ± 0.6	19.0 ± 0.9	18.9 ± 1.4	0.30
C-MN	146.4 ± 2.8	146.1 ± 3.5	148.8 ± 3.6*	0.0064
C-SD	5.6 ± 0.9	5.7 ± 0.7	5.6 ± 0.6	0.79
S-MN	147.5 ± 4.7	145.4 ± 5.0*	146.4 ± 6.3	0.048
S-SD	10.7 ± 1.2	11.0 ± 1.0	10.8 ± 1.2	0.53

* Significantly different from corresponding value at T = 0h (P < 0.05)
*** Significantly different from corresponding value at T = 0h (P < 0.001)
Abbreviations: mean (MN), standard deviation (SD), volume (V), conductivity (C), scatter (S).

Results

A total of 12 healthy subjects, 6 males and 6 females, were included in the study. Subjects were lean, normotensive and showed a normal lipid profile, leukocyte concentration and automated 5-part leukocyte differentiation (Table 2). Plasma TG increased by 0.91 ± 0.53 mmol/l (P < 0.001) four hours postprandially and returned to baseline at T = 8h. Total leukocyte count and absolute monocyte count were significantly increased

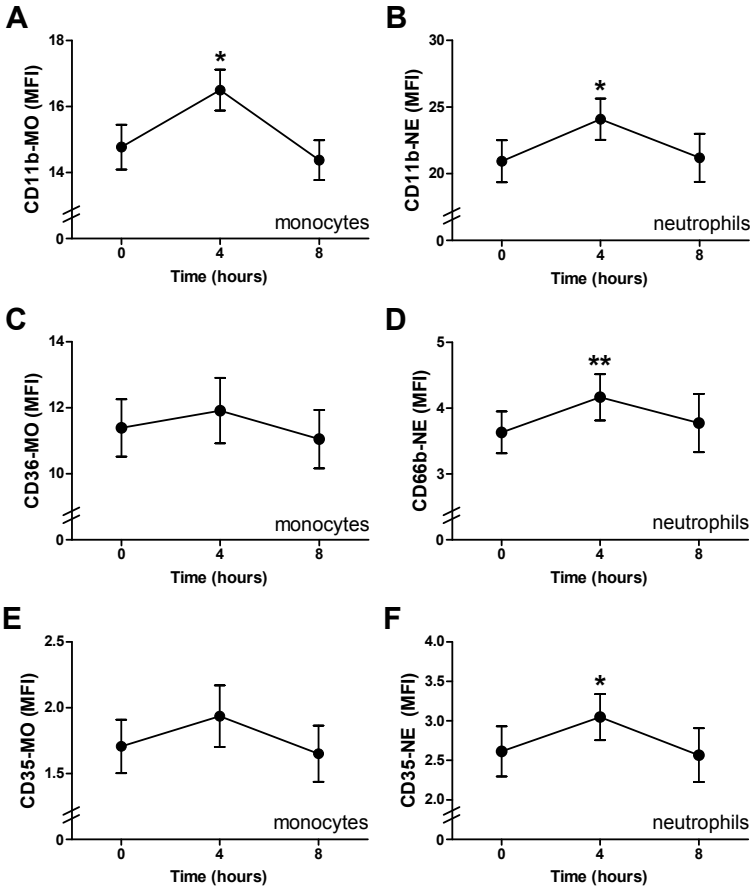

Figure 1: The mean (± SEM) changes in activation markers of monocytes and neutrophils after the ingestion of a standardized oral fat loading test (n = 12): Expression of CD11b by monocytes (A) and neutrophils (B) was significantly increased postprandially (P-ANOVA 0.004 and 0.024, respectively). CD36 expressed by monocytes (C) remained unchanged, but the degranulation marker CD66b increased significantly on neutrophils (D) postprandially (P-ANOVA 0.007). Furthermore, CD35 was significantly more expressed by monocytes (E) and neutrophils (F) postprandially (P-ANOVA 0.021 and 0.016, respectively). Abbreviations: cluster of differentiation (CD), monocytes (MO), neutrophils (NE). *P < 0.05 and **P < 0.01 when compared to T = 0h.

at the end of the OFLT, whereas absolute lymphocyte count gradually increased during the OFLT. The absolute neutrophil count increased gradually during the OFLT, but did not reach statistical significance (Table 3). The expression of CD11b on monocytes and CD11b, CD66b and CD35 on neutrophils increased significantly at T = 4h and declined thereafter. In addition, monocytes showed a trend of a postprandial increase in CD35 expression, whereas the expression of CD36 by monocytes remained unchanged (Figure 1).

The mean volume of monocytes increased significantly from 162.4 ± 4.3 a.u. to 165.0 ± 4.8 a.u. at t = 4h ($P < 0.001$) and returned to 163.5 ± 4.2 a.u. at T = 8h (P-ANOVA 0.0008). The mean volume of lymphocytes and neutrophils remained unchanged postprandially. The mean conductivity of lymphocytes, monocytes and neutrophils increased at the end of the OFLT (T = 8h) (Table 4). The mean scatter of neutrophils decreased significantly from 147.5 ± 4.7 to 145.4 ± 5.0 ($P < 0.05$) at T = 4h and returned to 146.4 ± 6.3 at T = 8h (P-ANOVA 0.048). The mean scatter from lymphocytes and monocytes remained unchanged postprandially. No significant postprandial changes were observed in the standard deviations of the VCS-data.

At T = 4h, CD11b expression on neutrophils was negatively correlated with the mean scatter of neutrophils (Pearson's r: -0.677, $P = 0.016$) and CD36 expression on monocytes was positively correlated with the mean conductivity of monocytes (Pearson's r: 0.606, $P = 0.037$). No other correlations were found between activation markers on monocytes and neutrophils with corresponding VCS-parameters at the postprandial peak.

Discussion

To our knowledge this is the first report on postprandial changes in leukocyte cell population data. Our study illustrates that leukocyte cell population data are influenced by non-infectious inflammatory conditions like physiological postprandial leukocyte activation. As reported earlier [5,6], postprandial leukocyte activation was confirmed by an increased expression of CD35, CD11b and CD66b on neutrophils and CD11b on monocytes at the postprandial peak. Postprandial TG increased by 0.91 mmol/l, which is comparable with a physiological diurnal increase in TG, since TG normally increase approximately 0.5-1.0 mmol/l during the day [16].

The mean monocyte volume was increased four hours postprandially. This result is in concordance with others who reported increased mean volume of monocytes in bacteremia [10,15], fungemia [15], hepatitis B infection [17] and malaria infection [14]. Our study also observed a postprandial decrease in mean scatter of neutrophils, which correlated with the postprandial expression of CD11b on neutrophils. These results were expected since neutrophils become activated postprandially. Similar changes in mean

scatter of neutrophils, albeit with a stronger magnitude, have been observed during systemic infection or sepsis [8,15] and after activation of neutrophils with granulocyte colony-stimulating factor (G-CSF) [18]. Neutrophil activation leads to degranulation and thus to a decrease in mean scatter. Upon activation, the secretory vesicles of neutrophils are fused with the plasma membrane and are exocytosed. These secretory vesicles are the main reservoir for CD35 and membrane proteins CD11b and CD66b [19]. Therefore, leukocyte activation leads to increased membrane expression of CD11b, CD66b and CD35.

The observed postprandial changes in mean volume of monocytes and mean scatter of neutrophils we observed were 1.7% and 1.4% compared to reported changes of 4.1 - 11.2% in infectious conditions [9,10,14,15]. The postprandial state may theoretically influence the usability of leukocyte cell population data as a diagnostic tool for infection detection. However, the postprandial changes in leukocyte cell population were rather small, which probably limits the clinical relevance of these postprandial changes in mean volume of monocytes and mean scatter of neutrophils. Nevertheless, our results confirm that leukocyte cell population data are already affected by a physiological postprandial inflammatory reaction. We hypothesize that these morphological changes are due to internalization of TG-rich lipoproteins by monocytes and neutrophils [5,7,20]. The question remains whether subjects with hypertriglyceridemia or diabetes will show similar changes in postprandial leukocytes.

A left shift of neutrophils may occur with the presence of band forms and immature neutrophils during an acute bacterial infection, which can lead to an increased standard deviation in volume of neutrophils [9,15]. However, here we did not observe any postprandial changes in the standard deviation of VCS parameters, nor did the LH750 flag any of the samples for immature neutrophils. We did observe a significant increase in total leukocyte count, lymphocyte and monocyte count during the OFLT, whereas neutrophils tended to increase postprandially without reaching statistical significance. These results are in concordance with previous reports, which showed increases in leukocyte count and absolute lymphocyte and neutrophil counts during comparable experiments [2,3,21]. Recently lymphocyte counts were shown to decrease one to two hours after a light mixed meal with normalization at four hours postprandially [22]. Here we have studied four and eight hours postprandially aimed at detecting leukocyte activation postprandially. We observed an increase in lymphocyte count, especially eight hours postprandially. Therefore, we could have missed an initial decrease in lymphocyte counts during the first two to four hours of the OFLT. Comparing both studies is difficult since different meals were used (a mixed meal versus a fat load) and the addition of glucose to a fat load has been shown to reduce the postprandial increase in TG [23].

In conclusion, monocytes and neutrophils of healthy adults become activated after the ingestion of a fat-containing meal, which temporarily affects leukocyte cell popula-

tion data in a similar way as in certain infectious diseases. However, the magnitude of these changes is limited. Therefore, the postprandial changes in leukocyte cell population data probably do not affect the clinical use of leukocyte cell population data as a diagnostic tool for infection detection.

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