Leukocytes and complement in atherosclerosis

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a. Leukocyte activation by triglyceride-rich lipoproteins

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ABSTRACT

Introduction: Leukocyte activation is an obligatory phenomenon in atherosclerosis. Postprandial lipemia has been closely linked to both atherosclerosis and inflammation. We studied leukocyte activation *in vivo* and *in vitro* by triglyceride-rich particles.

Methods: The leukocyte expression of CD11B and CD66B on leukocytes was evaluated during an Oral Fat Loading Test (OFLT) using flowcytometry. Incubations of freshly isolated leukocytes with chylomicrons (TRL) and lipid emulsions (ATRL) were carried out in the presence of a scavenger of oxidative stress, DMTU. Binding of apolipoprotein B (apoB) to leukocytes was studied by flowcytometry. The *in vivo* leukocyte uptake of TRLs was investigated by quantitating labeled dietary fatty acids in postprandial leukocytes.

Results: OFLTs (6 hrs) in 20 healthy subjects showed that monocyte CD11B increased by 33% and neutrophil CD11B and CD66B by 68% and 24% respectively, ($P<0.005$ for each). Neutrophils co-incubated with physiological concentrations of TRLs showed 62% increased CD11B ($P<0.005$) but CD66B did not change. Monocytes showed a significant dose dependent increased CD11B expression up to a maximum of 84%. Incubations with ATRLs in the hypertriglyceridemic range showed a 5-fold increased monocyte CD11B, which was even higher than induced by fMLP, and a dose dependent 2 to 3-fold increase of neutrophil CD11B and CD66B. DMTU decreased only the neutrophil CD66B expression by 36% ($P<0.05$). ApoB on leukocytes from 65 different subjects measured by flowcytometry was highest on neutrophils (40.5±22.8 au) compared to monocytes (15.5±11.0 au) and lymphocytes (4.3±4.3 au) ($P<0.001$ by ANOVA) suggesting preferential adherence of apoB-containing lipoproteins to neutrophils and monocytes. Postprandial experiments in 11 volunteers showed a concentration of ~2 umoles TG/L in leukocytes, which became enriched with meal-derived fatty acids ((1-^{13}C)16:0) suggesting uptake of exogenous fatty acids in the bloodstream.

Conclusions: Acute hypertriglyceridemia is a leukocyte activator most likely by binding of apoB-TRLs to leukocytes and uptake of fatty acids in the bloodstream. Although the total transport capacity of TRLs by leukocytes is limited, TG-mediated leukocyte activation could be an important pro-inflammatory and pro-atherogenic mechanism of hypertriglyceridemia associated to the generation of oxidative stress.

INTRODUCTION

Cardiovascular disease (CVD) is a major cause of death in the world. Atherosclerosis is considered a low-grade chronic inflammatory condition initiated by resident and recruited leukocytes in response to endothelial injury (1). Leukocyte count is positively related to CVD incidence and mortality (2) as well as to traditional cardiovascular risk factors, such as smoking, hyperlipidemia and insulin resistance (3,4).

Triglycerides (TG) and glucose are able to induce leukocyte activation, as has been shown *in vitro* (5,6) and *ex vivo* in hypertriglyceridemic patients (7). Neutrophil counts increase postprandially in healthy subjects, in CVD patients and in type 2 diabetics when TG and glucose rise (8-11). This neutrophil increase is associated to the production of pro-inflammatory cytokines and oxidative stress and it has been suggested that these changes may contribute to endothelial dysfunction (8-11).

Postprandial lipemia has been associated with the upregulation of leukocyte activation markers CD11b and CD66b in healthy volunteers and in patients with premature atherosclerosis (12,13). CD11b (also termed MAC-1 or CR3) is one of the most important markers involved in early adhesion of leukocytes to the endothelium (14,15). CD66b (also termed CEACAM8) is a degranulation marker of neutrophils and is not expressed on lymphocytes and monocytes (16). Increased expression of these markers on fasting leukocytes in patients with CVD and diabetes has also been described (13,17-21). Fasting leukocytes of patients with CVD have an increased lipid content when compared to controls and it was suggested that this was due to the uptake of chylomicrons in the bloodstream (22). Finally, leukocytes are able to internalize retinyl esters, as markers of intestinally derived TG-rich lipoproteins (TRLs) (23). Hence, postprandial inflammatory changes may involve leukocyte activation by uptake of TRLs leading to increased adhesion to and activation of endothelial cells. As a result migration of inflammatory cells and lipoproteins to the subendothelium may be enhanced, facilitating the atherosclerotic process.

To further elucidate the interaction between TRLs and leukocytes, we investigated the effect of postprandial TRLs on the *in vivo* and *ex vivo* activation of leukocytes by measuring the expression of the leukocyte activation marker CD11b and the neutrophil specific CD66b.

MATERIALS AND METHODS

Subjects

For the oral fat loading tests (OFLTs) in Utrecht two groups of normolipidemic subjects were included. Firstly, twenty healthy subjects (mean age 50±5 years, mean BMI 25±2 kg/m^2, waist circumference 0.92±0.10 m, HOMA index 0.77±0.74) with normal fasting lipids and glucose were selected to evaluate leukocyte activation 6 hours postprandially (short-term study).

Secondly, a different group of normolipidemic non-diabetic volunteers (mean age 51±4, mean BMI 26±2, waist circumference 0.94±0.07, HOMA index 1.49±0.88) were selected for 10 hours postprandial leukocyte activation studies (prolonged study).
In Oxford, eleven healthy volunteers (mean age 40±11 years, mean BMI 26.0±4.2 kg/m^2, waist circumference 0.89±0.10 m, HOMA index 1.90±0.98) were recruited by advertisement.
All subjects had normal fasting plasma cholesterol (<6.0 mmol/l), apolipoprotein B (apoB) (<1.0 g/l), TG (<2.0 mmol/l) and HDL (>1.0 mmol/l) levels.
In order to investigate apoB on leukocytes 65 patients (mean age 48.9 ± 11.4 years, mean BMI 26.5 ± 4.4 kg/m^2, waist circumference 0.86±0.11 m, HOMA index 1.42±1.3, total cholesterol 5.4±1.3 mM, HDL 1.08±0.31 mM, TG 1.67±0.93 mM, apoB 1.00±0.27 g/L, glucose 5.6±1.4 mM) were recruited in the outpatient lipid clinic of the Department of Internal Medicine in Utrecht. These patients visited our clinic for screening and treatment of risk factors for atherosclerosis.
The Independent Ethics Committee of the Institutional Review Board of the University Medical Center Utrecht and Oxfordshire Clinical Research Ethics Committee (Oxfordshire, UK) approved the study. The participants gave written informed consent.

Study meals and design

Fresh cream was used for the OFLTs in Utrecht. This is a 40% (w/v) fat emulsion with a poly-unsaturated/saturated fat ratio of 0.10, containing 0.001% (w/v) cholesterol and 3% (w/v) carbohydrates, representing a total energy content of 3,700 kcal/L. Cream was ingested at a dose of 50 g fat per m^2 body surface.
The Oxford meal contained 40 g carbohydrate (Rice Krispies, Kellogs Co Ltd, UK) and 40 g fat in the form of a warm chocolate drink. The fat consisted of 40 g macadamia oil (Olivado, New Zealand), chosen because of its high content of palmitoleic acid, 16:1*n*-7. To this 250 mg of (1-^{13}C)palmitic acid was added to trace dietary fatty acids.
Venous blood was obtained after a 12 hours overnight fast. For the OFLTs, a cannula was placed for venous blood sampling. The subjects rested for one hour before ingesting the test meal. During the tests, the participants remained supine and were only allowed to drink mineral water. Peripheral blood samples were obtained in sodium EDTA (2 mg/mL) before (t=0) and at regular time intervals up to 6 hours. For the experiments in Oxford blood samples were collected into lithium heparin tubes before and at 4 h after ingestion.
For the *ex vivo* experiments, chylomicrons from the same control were isolated from blood samples (obtained in Na-Heparin tubes) taken 4 hours postprandially after the OFLT.

Analytical methods

Total cholesterol, HDL obtained after precipitation with phosphotungstate/$MgCl_2$ and TG were measured in duplicate by colorimetric assays (Roche diagnostics, Germany). (8-11). Glucose was

measured by dry chemistry colorimetry (Vitros 250; Johnson & Johnson, Clinical Diagnostics, Rochester, NY, USA) (8-11). Total plasma apoB was quantitated by immunoturbidimetry (9-11). Native TRLs (NTRLs) were obtained by means of ultracentrifugation as described previously in detail (24).

Ex vivo leukocyte activation

Samples for the determination of expression of leukocyte activation markers were stored at 4°C. In order to minimize *ex vivo* leukocyte activation we found in preliminary studies that using an open system of blood sampling, incubating in Fluorescence Activated Cell Sorter (FACS) tubes with NaCl 0.9% added for volume correction, resulted in the least *ex vivo* activation in the 'basal' (control) situation (data on file).
FACS tubes were filled with 400 µL blood containing the appropriate buffers (12,13). Experiments were carried out by incubating whole blood with low concentrations of NTRLs and high concentrations of artificial TRLs (ATRL, Lipofundin 20%; Braun, Melsungen, Germany). The experiments were also carried out with different concentrations of glucose (BDH Chemicals Ltd, Pool, England). The concentrations of TG were 1.5 (which was the fasting plasma TG concentration), 1.66, 1.85 and 2.1 mM, respectively for NTRL, and 1.5, 10.0 and 15.0 mM for ATRL. Glucose concentrations were 4.0, 10.0 and 15.0 mM. Dimethylthiourea (DMTU; Janssen Chimica, Beerse, Belgium) 10.0 mM was used as scavenger of reactive oxygen species (ROS). As positive control N-formylmethionyl-leucyl-phenylalanine (fMLP; Sigma, St Louis, USA) was used. In order to equalize the volume of the tubes pyrogen free NaCl 0.9% infusion solution (Baxter B.V; Utrecht, the Netherlands) was used. All *in vitro* experiments were done each time using whole blood of one untreated healthy lean volunteer. All investigated incubation conditions were performed in six-fold. The tubes were shaken gently in a warm water bath at 37° C for 15 minutes. After this incubation, tubes were placed on ice with the purpose to stop *ex vivo* leukocyte activation. Subsequently, the tubes were prepared for the analysis of expression of activation markers on the leukocytes

Leukocyte activation markers

Using fluorescent labeled monoclonal antibodies (MoAbs), the cell surface expression of one pair of leukocyte activation markers was detected by direct immunofluorescence in duplicate and evaluated by flowcytometry: a combination of fluorescein isothiocynate (FITC) conjugated CD66b (CLB, Amsterdam, the Netherlands) and phycoerythrin (PE) conjugated CD11b (DAKO, Denmark) (12,13,21). The flowcytometric analysis has been described in detail (12,13,21). To avoid *in vitro* activation, the leukocytes were incubated with MoAbs in whole blood at a saturating concentration of 1:10 for 30 minutes in the dark on ice. Erythrocytes were lysed by adding 1 mL of ice-cold isotonic erythrocyte lysing solution (NH_4Cl 0.19 M; $KHCO_3$ 0.01 M; $Na_2EDTA{\bullet}2H_20$

0.12 M, pH 7.2) for approximately 15 minutes and centrifuged at 500 x *g* for 5 minutes at 4 °C. The remaining leukocyte suspension was washed twice in ice-cold PBS supplemented with bovine serum albumin (BSA 0.2%). Within one hour a total of 5000 cells/sample was analyzed by flowcytometry (Becton Dickinson) and CellQuest software (12,13,21). Neutrophils, lymphocytes and monocytes were identified by their characteristic forward and side scattering properties. Fluorescence intensity of each cell was expressed as the average mean fluorescence intensity (MFI) of the duplo, given in arbitrary units (au). In each experiment the series of postprandial measurements were performed with identical instrument settings and all were carried out in Utrecht.

Postprandial fatty acid changes in leukocytes

Leukocytes were prepared by lysing red blood cells from 7 mL whole blood using 21 mL red blood cell lysis solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA). The cells were pelleted by centrifugation at 4°C (217 x *g* for 10 min), and washed three times with saline (0.9% w/v NaCl). Cells were stored at -20°C before analysis. Blanks were also prepared using red blood cell lysis solution and saline. Heparinised plasma was also prepared and stored at -20°C before analysis. Lipids were extracted from cells and from plasma by the method of Bligh & Dyer (25) and the TG fraction was isolated by thin-layer chromatography. Fatty acid methyl esters were prepared by transesterification with methanolic sulphuric acid and the fatty acid composition was analysed by gas chromatography (GC) as described previously (26). ^{13}C enrichment in TG-palmitic acid was assessed using GC-combustion-isotope ratio mass spectrometry (Thermo-Electron Delta Plus XP, Bremen, Germany). All analyses were made in triplicate in Oxford.

Apolipoprotein B on leukocytes

Blood was washed once with PBS/BSA 0.2%. Blood cells were incubated with a goat-antihuman polyclonal antibody at a dilution of 1:25 anti-apolipoprotein B (Chemicon, USA), for 30 minutes on ice. This antibody recognizes both apoB48 and apoB100. Goat serum (Dako, Denmark) served as an isotype control for a-specific staining. After a washing step, cells were incubated with 1:10 rabbit anti-goat Ig-FITC (Dako, Denmark) for 30 minutes on ice. Unbound antibodies were washed with PBS/BSA 0.2% and erythrocytes were lysed by adding ice-cold isotonic lysing solution for approximately 15 minutes. After lysis, the leukocytes were washed once with PBS/BSA 0.2%. Samples were kept in the dark and on ice until analysis. A total of 5000 cells/sample was analyzed by flowcytometry.

Statistics

Data are given as mean ± SD in the text and in the figures. Time series analysis were carried out using repeated measures ANOVA with Bonferroni correction and LSD test as post hoc test or paired t-test where indicated. Wilcoxon test was used for paired comparisons of non-parametric variables (fatty acid leukocyte content). For statistical analysis SPSS version 14.0 was used. *P* values less than 0.05 (2-tailed) were considered statistically significant.

RESULTS

Postprandial leukocyte and TG changes (Figure 1)

OFLTs showed that TG increased significantly between t=2 to t= 6 hours, with a maximum at t=4 hours in both Utrecht groups. There was a postprandial increase in leukocyte count. This increment was due to the neutrophil and lymphocyte increase, being maximal at t=3 hrs. Monocytes did not change postprandially.
In both, short-term and the prolonged studies, neutrophil CD11b expression increased after two hours, followed by a gradual increase to a maximum at t=6 hrs (+87% and +76% vs. t=0 hrs, respectively, P<0.005) (Figure 1A). Hereafter, neutrophil CD11b remained increased in the prolonged study up to t=10 hrs (+71% vs. t=0 hrs, P<0.05) (Figure 1A). In the short-term study, monocyte CD11b expression showed a trend after one hour and a late 6 hours increase (Figure 1B). In the prolonged study, monocyte CD11b showed a gradual increase with a maximum on t=10 hrs (+37% vs. t=0 hrs, P<0.005) (Figure 1B). Neutrophil CD66b also showed a late response after 6 hours in the short-term study (Figure 1C). In the prolonged study, this increase reached its peak at t=10 hrs (+25% vs. t=0 hrs, P<0.005). The expression of CD11b on lymphocytes was low and did not change postprandially (data not shown).

Ex vivo leukocyte activation (Figures 2-4)

The plasma TG content used for these experiments was 1.5 mM. Raising the TG content by 0.35 mM with NTRLs increased the neutrophil CD11b expression (308 ± 52 au vs. 499 ± 22 au, respectively; P <0.005, Figure 2A). Addition of higher concentrations of NTRLs did not show any significant changes. The expression of monocyte CD11b showed a significant dose dependent response after incubation with increasing concentrations of NTRLs (294 ± 23 au, 348 ± 48 au, 492 ± 13 au, 539 ± 15 au by ANOVA P <0.05, Figure 2A). Whole blood incubations with increasing doses of TRLs did not lead to a significant effect on the expression of CD66b (Figure 2B).
High concentrations of ATRLs increased CD11b expression by 4.4 and 4.5 times on monocytes and by 1.9 and 3.3 times on neutrophils (P<0.005 for each, Figure 3A). The ATRL-induced

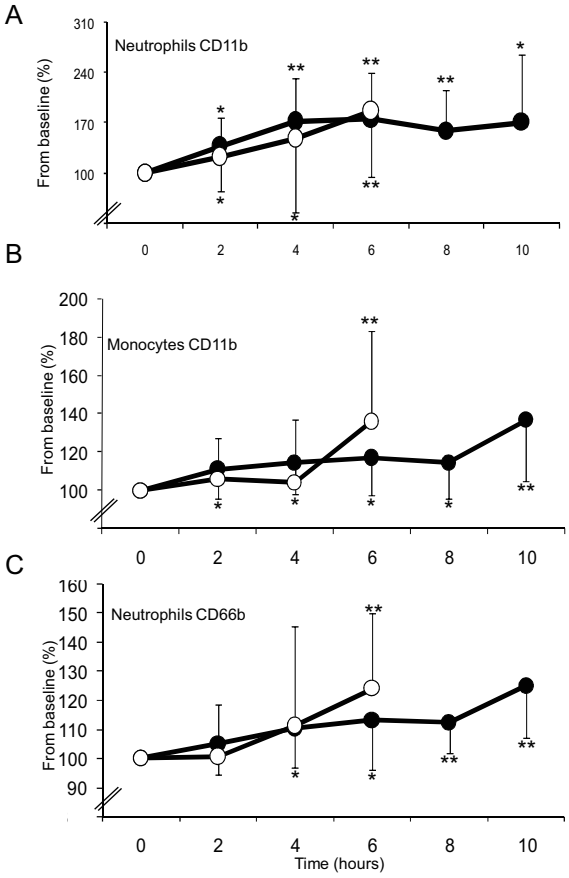

Figure 1. Mean±SD expression of CD11b on neutrophils (1A) and monocytes (1B) and CD66b expression on neutrophils (1C) after a 6 hours oral fat load in 20 healthy subjects (open circles) and after a 10 hours oral fat load in another group of 12 normolipidemic subjects (closed circles). Expression of the markers is shown as Mean Fluorescence Intensity (MFI). *: P<0.05, **: P<0.005, both compared to t=0

monocyte CD11b expression was higher than the CD11b expression induced by fMLP (1170 ± 33 and 1210 ± 42 vs. 703 ± 48 au; P <0.005). The CD66b expression on neutrophils showed a gradual rise by increasing TG concentrations (1.5 and 2.1 times higher than control; P<0.005, Figure 3B).

Incubations with increasing concentrations of glucose did not change the expression of leukocyte activation markers.

DMTU blunted the ATRL-induced neutrophil CD66b expression (37% lower expression when compared to ATRL alone, P<0.05, Figure 4C). However, ATRL- induced CD11b expression on monocytes and neutrophils was not changed by DMTU (4A and B).

CD11b expression on lymphocytes was low and did not change by incubation with any of the additives and DMTU had no significant effect (data not shown).

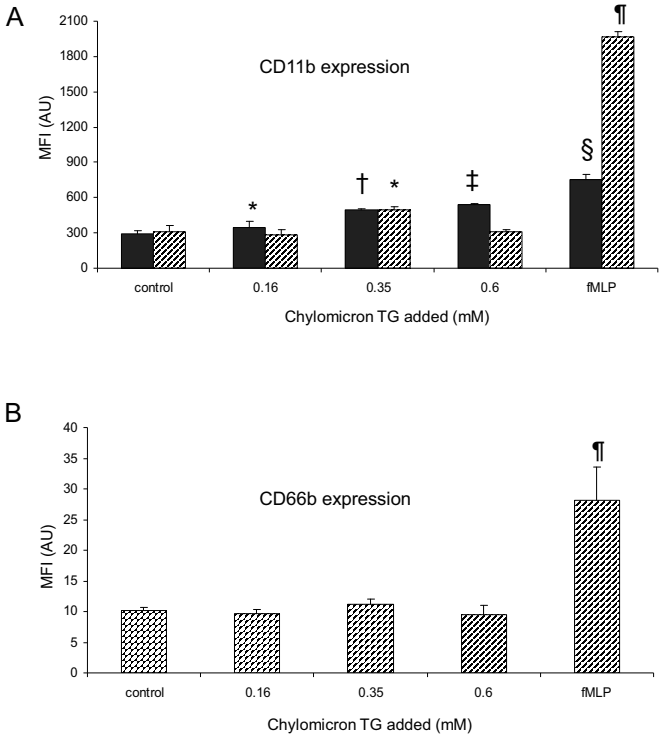

Figure 2. Mean±SD expression of CD11b on neutrophils (▨) and monocytes (■) (2A) and CD66b expression on neutrophils (▨) (2B). 15' incubation in 37° C in six-fold with increasing concentrations of NTRLs (final concentrations in the samples 1.66, 1.85 and 2.10 mM). fMLP 10-4 mM was the positive control. Pyrogen free NaCL 0.9% infusion solution (Baxter BV, Utrecht, the Netherlands) was used to equalize the volumes. Expression of the markers is shown as Mean Fluorescence Intensity (MFI). *: P<0.05 vs. control. †: P<0.05 vs. 1.66 mM. ‡: P<0.05 vs 1.85 mM. §: P < 0.05 vs. 2.1 mM. ¶ :P<0.005 vs. 2.1 mM.

Fatty acid changes in postprandial leukocytes

A small amount of TG was detectable in leukocytes isolated from fasting blood. This averaged 2.1 ± 1.2 µmol/L, compared with plasma TG concentration of 1340 ± 720 µmol/l. In order to show that this TG did not arise from contamination with plasma, the fatty acid composition was compared with the composition in plasma from the same donor. Comparing 10 major fatty acids, the composition of leukocyte TG was significantly different from that of plasma ($P < 0.001$, repeated measures ANOVA). Notably, the proportion of palmitic acid, 16:0, was lower in leukocytes than in plasma (15.9 ± 3.1 *vs.* 27.4 ± 1.1 g/100g); that of stearic acid, 18:0, was markedly higher in leukocytes (23.4 ± 2.9 *vs.* 2.9 ± 0.2 g/100g); and linoleic acid, 18:2*n*-6, was lower in leukocytes (7.7 ± 1.9 *vs.* 18.4 ± 2.4 g/100g).

After the mixed meal, the plasma TG rose from 1.34 ± 0.72 mM (fasting) to 2.01 ± 1.22 mM at 4 h (P = 0.02). The total TG content of leukocytes did not increase but the fatty acid composition

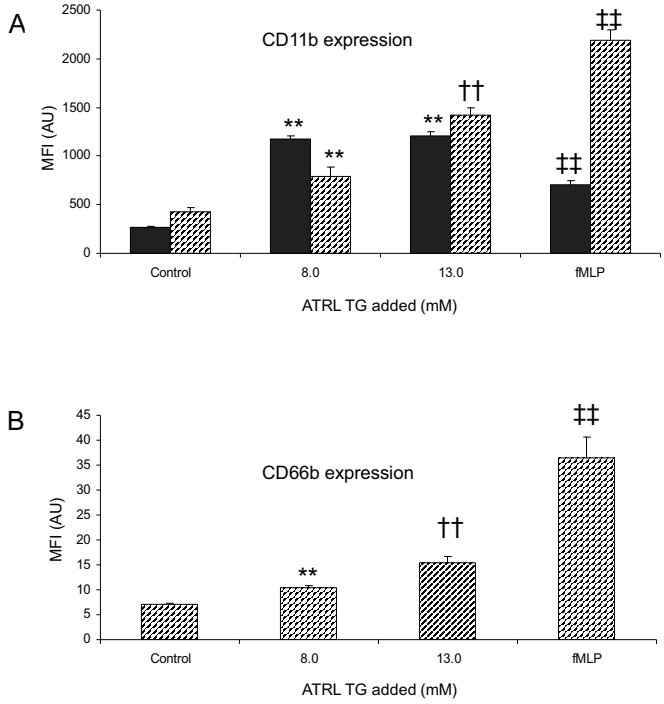

Figure 3. Mean±SD expression of CD11b on neutrophils (▨) and monocytes (■) (3A) and CD66b expression on neutrophils (▨) (3B). 15' incubation in 37° C in six-fold with increasing concentrations of ATRLs (final concentrations in the samples 10 and 15 mM). fMLP 10-4 mM was the positive control. Pyrogen free NaCL 0.9% infusion solution was used to equalize the volumes. Expression of the markers is shown as Mean Fluorescence Intensity (MFI). **: P<0.005 vs. control. ††: P<0.005 vs. 10 mM. ‡‡: P<0.005 vs. 15 mM.

changed. Leukocyte TG became enriched with the 'marker' fatty acid in the meal, 16:1*n*-7 (1.9 ± 0.8 g/100g to 4.9 ± 1.5 g/100g, $P = 0.05$, Wilcoxon test) and depleted in the endogenous fatty acid 18:0 (23.4 ± 2.9 g/100g to 17.2 ± 2.1 g/100g, $P = 0.02$, Wilcoxon test). There was also a significant increase in leukocyte TG-palmitic acid (^{13}C) enrichment ($P = 0.004$).

ApoB on leukocytes (Figure 5)

ApoB was detected on all leukocyte cell types. ApoB on neutrophils was significantly higher (40.5 ± 22.8 au; P< 0.0001) than apoB on monocytes (15.5 ± 11.0 au; P< 0.001) and lymphocytes (4.3 ± 4.0 au; P< 0.001, Figure 5). The apoB signal did not change significantly in postprandial leukocytes (data not shown).

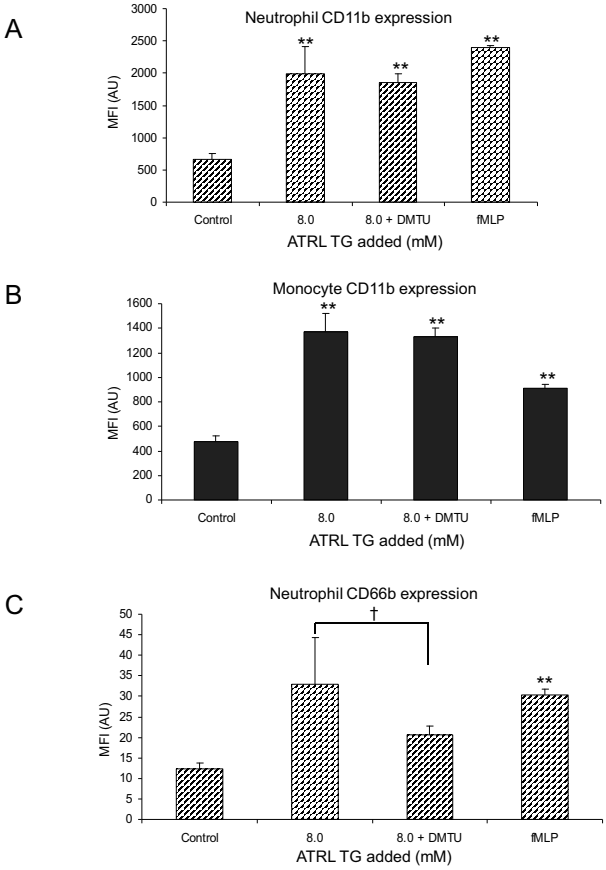

Figure 4. Mean±SD expression of CD11b on neutrophils (▨) and monocytes (■) (4A and B) and CD66b expression on neutrophils (▨) (4C). 15′ incubation in 37º C in six-fold with NTRL 10 mM, NTRL 10 mM plus DMTU 10 mM. fMLP 10-4 mM was the positive control. Pyrogen free NaCL 0.9% infusion solution was used to equalize the volumes. Expression of the markers is shown as Mean Fluorescence Intensity (MFI). **: P<0.005 vs. control †: P<0.05 vs.10 mM ATRLs.

DISCUSSION

This is the first study showing that TRLs induce early monocyte and neutrophil activation *ex vivo* at hypertriglyceridemic conditions, while physiologic changes of TRLs results only in early monocyte activation in a dose dependent manner. Furthermore, the data suggest that oxidative stress is involved in high TG-induced early activation and degranulation of neutrophils. The uptake of meal-derived fatty acids in postprandial leukocytes was also demonstrated. Finally, binding of TRLs to leukocytes was supported by the presence of apoB on neutrophils and monocytes. Surprisingly, triglycerides seemed to activate monocytes better than fMLP.

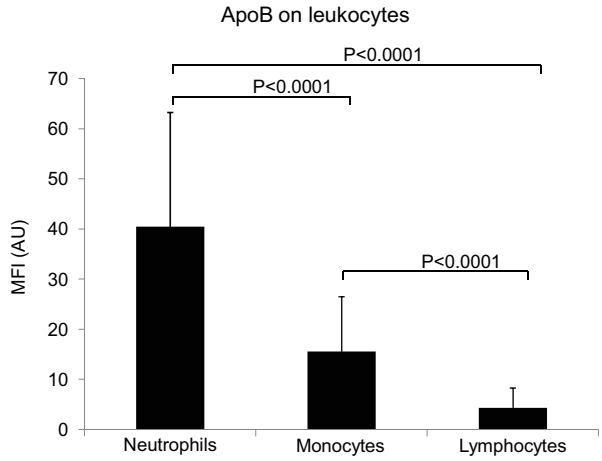

Figure 5. Mean±SD expression of apolipoprotein B on neutrophils, monocytes and lymphocytes in 65 patients of our clinic for screening and treatment of risk factors for atherosclerosis. Expression of apoB is shown as Mean Fluorescence Intensity (MFI).

Apparently, TRLs are very potent activators of monocytes. Future studies should address the exact molecular mechanisms of TG-mediated monocyte activation.

Furthermore, besides showing 6 hours postprandial leukocyte upregulation, leukocyte activation *in vivo* was also observed in normolipidemic volunteers up to 10 hours. Essentially, there were no differences in leukocyte activation between the short-term (6 hours) and the prolonged study (10 hours). One of the interesting points of this study were the differences between the rather late upregulation of CD11b and CD66b in the *in vivo* study and the rapid upregulation of these markers in the *in vitro* experiments. After ingestion of fat leukocytes and TG start to increase after 1 and 2 hours, respectively. In theory, this should be the moment of activation of the leukocytes. In our study this occurred later. An explanation could be that leukocyte activation in theory leads to adherence to the endothelium, resulting in underestimation of the *in vivo* situation by peripheral blood sampling. Supporting this hypothesis, it has been shown that angioplasty-induced expression of leukocyte activation markers is higher on locally obtained cells than on cells in peripheral blood (27). Alternatively, *in vivo,* protecting mechanisms possibly derived from sources like the endothelium may be operational. Nitric oxide is such a protecting factor (28). Further studies on the intra-individual sites of activation are needed to explore these differences.

The effects of TRLs on endothelial cells were not studied, but it has been shown that postprandial hypertriglyceridemia induces acute endothelial dysfunction (9,29,30), and that leukocytes may be involved (9). Other studies have suggested that hypertriglyceridemia (31) and nonesterified fatty acids (33) lead to binding of higher number of monocytes to the endothelium, and that HMG-CoA reductase inhibitors are able to reduce this increased adhesiveness (34).

In our study, early glucose-mediated activation was not observed. Activation of leukocytes by glucose has been shown *in vitro* using monocyte cell-lines (34,35). However, in these studies, cell activation was established by gene expression and the production of cytokines and NF-kappaB. *In vivo* leukocyte activation induced by glucose has also been shown with oral glucose tolerance tests (10). The present study suggests that short-term leukocyte activation by glucose is less likely than by triglycerides, which is in agreement with previous observations (9).

Although there is evidence that a subset of lymphocytes expresses CD11b and that it may play a role in the migration of these cells (36), in our experiments lymphocytes did not show significant CD11b changes. This is in agreement with postprandial studies in which lymphocytes did not show a meal dependent increase in cell count and activation (8,12,13,21) in contrast to the effects on neutrophils and monocytes.

Earlier reports have indicated that minimally modified apoB-containing lipoproteins (37-39) and postprandial low-density lipoprotein (LDL) particles (40) can activate monocytes. The mechanism of this activation was not elucidated. Wanten et al. have shown that TRLs induce ROS production in human neutrophils enhancing the respiratory burst of these cells (5,6).

Others have suggested that also monocytes are able to generate ROS under similar conditions (41,42). To the best of our knowledge, the role of oxidative stress in the acute TRL-induced CD11b and CD66b expression has not been investigated before. In our experiments, DMTU suppressed TRL-induced activation of CD66b on neutrophils, but it did not change the neutrophil or monocyte CD11b expression. These data may suggest that oxidative stress is involved in the process of acute degranulation of neutrophils, but not in adhesion of monocytes and neutrophils to the endothelium. However, it has been shown that oxidative stress plays a role in the adhesion of monocytes to the endothelium since inhibitors of NADPH oxidase reduced the mRNA levels of CD11b after prolonged incubations during 48 hours (32). Our data suggest that early (<15 minutes) monocyte CD11b upregulation may be mediated by a different mechanism than oxidative stress.

Assessing fatty acid composition changes may be a more accurate estimate of changes in leukocytes than measuring the absolute concentration change, as it is known that the amount of postprandial leukocytes change upon fat ingestion (8,12,43). We are not aware of other experiments measuring quantitatively the postprandial fatty acid changes within the postprandial leukocytes. Although the total change and transport of TRLs by leukocytes is limited, this is the first study showing changes in fatty acid composition in leukocytes. Consequently, our data suggest direct uptake of exogenous fatty acids in the blood stream by leukocytes. The incorporation of extracellular fatty acids, either TG-fatty acids or non-esterified, into cellular TG has been shown previously in lymphocytes *in vitro* (44,45). The cellular TG content did not change significantly in this short postprandial period, so we presume that the cellular TG pool is continuously turning over. Extracellular fatty acid incorporation would thus lead to remodeling, as has been suggested previously for granulocyte membrane fatty acids (46). The presence of apoB on neutrophils and monocytes suggests binding of TRLs to these cells potentially

triggering leukocyte activation (especially monocytes and neutrophils). This phenomenon could initiate a cascade of inflammatory processes like the production of cytokines, activation of endothelial cells and adherence to the endothelium. Especially the latter is an obligatory step in the generation of the atherosclerotic plaque (1).

The mechanism of apoB binding to neutrophils and monocytes is unclear. One explanation could be that these leukocytes, which carry LDL receptors (47), bind TRL by the apoB moiety. However, lymphocytes have also LDL receptors (47,48), therefore a different mechanism may be involved. In our opinion, further studies should address this issue, but also investigate the clinical relevance of apoB binding to these cells.

It has been shown previously that postprandial leukocyte *increase* occurs in young, lean normolipidemic subjects (9), in healthy middle-aged subjects (12) and in mildly hyperlipidemic subjects with premature coronary artery disease (13). Furthermore, increased postprandial *activation* has also been reported before (12,13). In these studies age, BMI and HOMA were not determinants of this response. The current study not only confirms these findings, but also provides additional information on the mechanisms involved. Moreover, this is the first paper showing that leukocytes in humans *in vivo* interact directly with TRLs and are able to take up dietary fat.

Adhesion of leukocytes to the endothelium is a prerequisite for the development of atherosclerosis (49). Several studies have shown reduced plaque formation and prevention of endothelial dysfunction when adherence to the endothelium was prevented (50-52). Binding of TRLs to the leukocytes and opsonization, especially in the postprandial situation may be one of the first events in the activation of neutrophils and monocytes (53). Concomitant activation of endothelial cells may occur, making this the first step in the process of atherosclerosis already occurring in the bloodstream in contrast to the current view that atherosclerosis starts by the migration of LDL and TRL-remnants to the subendothelium (49,54-56).

A limitation in our *in vitro* study is the fact that we did not study the effects of potential platelet binding to the leukocytes. Platelets may bind to leukocytes resulting in a slight overestimation of the expression of the markers as has been reported elsewhere (57). So, platelet binding may be a confounding factor. In our experimental design this phenomenon most likely occurred in every incubation as has been shown before (57). Thus, in the case of TG-concentration and dose dependent enhanced expression, the increase would be caused by the TG and would not change our conclusions.

Another limitation is that we did not determine the degree of activation of the integrins. However, the expression of Mac-1 (CD11b) is a good marker for the rate of its activation. It has been shown that the activation of the integrin Mac-1 (8B2 neoepitope) occurs rapidly after coronary stenting, followed by CD11b expression (58). In another study, it has been shown that the upregulation of CD11b is paralleled by the activation of its integrin. Thus in the present study, we have measured CD11b, which is caused by the activation (57,58).

In conclusion, triglyceride-rich lipoproteins induce acute monocyte and neutrophil activation. Oxidative stress plays a role in acute TRL-induced neutrophil activation, but not in monocyte activation. The mechanism involves binding of apoB to neutrophils and monocytes and post-prandial intracellular incorporation of fatty acids.

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