Enhanced Fcγ Receptor I, $\alpha_M\beta_2$ Integrin Receptor Expression by Monocytes and Neutrophils in Rheumatoid Arthritis: Interaction with Platelets

ANDREIA BUNESCU, PETER SEIDEMAN, RODICA LENKEI, KLAS LEVIN, and NILS EGBERG

ABSTRACT. ***Objective.*** To investigate platelet and leukocyte activation and interaction in patients with rheumatoid arthritis (RA) and the effect of methotrexate (MTX) or anti-tumor necrosis factor-α (TNF-α) treatment on these variables.

Methods. Four-color flow cytometry analysis was performed for quantitative measurement of platelet (P-selectin, PAC-1) and leukocyte (CD11b, CD64) activation markers and estimation of percentage of leukocyte-platelet complexes in whole blood in 20 patients with RA before and after 6 weeks of therapy and in 20 controls. In addition, measures of soluble P-selectin (sP-selectin), ß-thromboglobulin, fibrinogen, prothrombin fragment 1+2, D-dimer, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), interleukin 6 (IL-6), and TNF-α and tender and swollen joint counts were carried out.

Results. Before therapy, PAC-1 binding, expression of CD11b and CD64 on monocytes and neutrophils, circulating levels of monocyte (CD11b+ or CD64+)-platelet complexes, monocyte-PAC-1+ platelet complexes, CRP, ESR, IL-6, TNF-α, fibrinogen, D-dimer and sP-selectin were significantly higher in RA patients compared to controls. The anti-TNF-α therapy significantly reduced levels of monocyte-PAC-1+ platelet complexes, sP-selectin, CRP, ESR, IL-6, TNF-α, fibrinogen, and D-dimer and tender and swollen joint counts. CD64 expression on monocytes was significantly decreased by MTX therapy. PAC-1 binding was not inhibited by MTX or anti-TNF-α.

Conclusion. Increased platelet and leukocyte activation and increased formation of leukocyte-platelet complexes in patients with RA suggest a status of simultaneous activation of the immune and hemostatic systems. (J Rheumatol 2004;31:2347–55)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
PLATELET
FLOW CYTOMETRY
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Rheumatoid arthritis (RA) is an autoimmune disease of synovial joints characterized by chronic inflammation and progressive destruction of cartilage and bone[1]. Cellular composition of the inflamed synovial tissue reveals an extensive infiltrate of T cells, monocytes, neutrophils, and plasma cells. Certain cytokines, e.g., interleukin 1 (IL-1), IL–6, IL-8, and tumor necrosis factor-α (TNF-α), are abundant in patients with RA and account for important processes in joint destruction, such as stimulation of production of metalloproteinases, synovial fibroblast proliferation, and influx of inflammatory cells[2]. Cell adhesion molecules involved in the pathogenesis of RA such as selectins (E, L, and P-selectin), $\alpha_M\beta_2$ integrin (Mac-1, CR3, CD11b/CD18), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1, and platelet endothelial cell adhesion molecule 1[3] are essential for interaction between platelets, leukocytes, and endothelial cells, resulting in leukocyte migration from the circulation into inflamed tissues. Binding of P-selectin expressed on activated platelets to P-selectin glycoprotein ligand-1 (PSGL-1) on monocytes induces tissue factor expression[4], which leads to fibrin formation, connecting the inflammatory process with coagulation.

Following platelet activation, glycoprotein (GP) IIb/IIIa undergoes a conformational change, which converts the complex into a functional receptor for fibrinogen, a process required for normal platelet aggregation[5]. PAC-1, a monoclonal antibody, binds only to activated platelets and detects this change in GPIIb/IIIa. Adhesion of activated platelets to endothelial cells is mediated via a platelet-GPIIb/IIIa bridging interaction involving platelet-bound fibrinogen, ICAM-1, and GPIbα on endothelial cells, and consequently may

From the Department of Clinical Chemistry, Capio Diagnostik, St. Göran's Hospital; Reumacenter, Sabbatsberg Hospital; and the Departments of Medicine and Clinical Chemistry, Karolinska Institute, Stockholm, Sweden.

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A. Bunescu, MD, Department of Clinical Chemistry, Capio Diagnostik, St. Göran's Hospital; P. Seideman, MD, PhD, Associate Professor, Reumacenter, Sabbatsberg Hospital, Department of Medicine, Karolinska Institute; R. Lenkei, MD, PhD, Flow Cytometry Laboratory, Capio Diagnostik, St. Göran's Hospital; K. Levin, MD, PhD, Department of Clinical Chemistry, Capio Diagnostik, St. Göran's Hospital; N. Egberg, MD, PhD, Associate Professor, Department of Clinical Chemistry, Karolinska Hospital.

Address reprint requests to Dr. A. Bunescu, Department of Clinical Chemistry, Capio Diagnostik, St. Göran's Hospital, 11281 Stockholm, Sweden. E-mail: Andreia.Bunescu@capio.se

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contribute to the formation of intravascular platelet aggregates[6]. The $\alpha_M\beta_2$ integrin on activated leukocytes binds firmly to ICAM-1 expressed on endothelial cells and to fibrinogen, leading to fibrinogen/fibrin accumulation at sites of inflammation[7]. The interaction between GPIbα on platelets and $\alpha_M\beta_2$ integrin mediates leukocyte-platelet adhesion and promotes inflammation at the thrombotic site[8]. The formation of leukocyte-platelet complexes occurs via P-selectin and its PSGL-1 on leukocytes, and their role may be that they represent targeting of both cell types at the inflammatory and hemostatic sites[9]. For example, adhesion of platelets to leukocytes via P-selectin was shown to induce the release of monocyte chemoattractant protein-1 (MCP-1), IL-8, and IL-1ß by leukocytes[10], which has been detected in the joints/serum of patients with RA[2,11]. The binding of activated platelets through P-selectin to PSGL-1 on leukocytes can also increase platelet aggregation[12]. Increased association of leukocytes with platelets has also been reported in patients with stable angina, unstable angina, myocardial infarction, and RA[13-16].

Receptors for the Fc region of IgG (FcγR), expressed by leukocytes and platelets and identified as FcγRI (CD64; on macrophages, monocytes, and neutrophils), FcγRII (CD32; on monocytes, neutophils, and platelets), and FcγRIII (CD16; on neutrophils, macrophages, and natural killer cells), participate in RA most probably by induction of phagocytosis, recruitment of inflammatory cells to the lesion, and clearance of immune complexes from circulation[17].

The aims of this pilot study were (1) to determine whether platelet activation (P-selectin, PAC-1 binding), leukocyte activation (CD11b, CD64), and their interaction (leukocyte-platelet complexes) is increased in patients with RA; (2) to determine the connection between the variables noted above and ß-thromboglobulin (ß-TG), soluble P-selectin (sP-selectin), fibrinogen, prothrombin fragment 1+2 (F1+2), D-dimer, disease activity indicators [swollen joint count, tender joint count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), IL-6, TNF-α)]; and (3) to determine whether treatment with methotrexate (MTX) or TNF blocker is associated with lower levels of platelet/leukocyte activation and leukocyte-platelet complexes.

MATERIALS AND METHODS

We studied 20 patients (15 women and 5 men, mean age 56 years, range 29–76) with RA according to the American College of Rheumatology criteria[18], and 23 healthy volunteers (18 women and 5 men, mean age 55 years, range 22–78) with no history of thrombosis, infection, or any rheumatic disease as controls. Patients were excluded from participation if they had any inflammatory condition other than RA, a history of deep vein thrombosis/pulmonary embolism or surgery within the last 3 months, were actively bleeding, had a known hemorrhagic diathesis, or had received glucocorticoids within the last 12 weeks or any nonsteroidal antiinflammatory drug or analgesics within 2 days prior to baseline visit. As classified by clinical disease activity (17 swollen and tender joints, ESR 25 mm/h), 2 groups of patients were studied. The first group was less active than the second group, which had > 17 swollen and tender joints and ESR > 25 mm/h. The first group (10 patients) was treated with MTX alone, 10–25 mg/week orally, and was defined as the MTX-T group. The second group, the TNF-T group, received infliximab (Remicade®), 3 mg/kg administered intravenously at 0, 2, 4, and 6 weeks (8 patients) or adalimumab (D2E 7, Abbott Scandinavia AB, Solna, Sweden), 40 mg administered subcutaneously every other week (2 patients). All patients taking infliximab were taking a stable dose of MTX (7.5–22 mg/week orally) for more than 3 months. The Ethics Committee of the Karolinska Hospital had given its approval of the study, and informed consent was obtained from all participants.

Blood sampling. Blood samples were taken on Day 0 before treatment and after 6 weeks of treatment. Venous blood was collected into a Vacutainer tube (Becton Dickinson, San Jose, CA, USA) containing lithium-heparin for measurement of CRP, IL-6, and TNF-α, and a mixture of EDTA, theophylline, and prostaglandin-E1 for ß-TG. A seditainer (Becton Dickinson) was used for ESR. For flow cytometric analyses, fibrinogen, D-dimer, and F1+2, blood was drawn without stasis into Vacutainer tubes (Becton Dickinson) containing 0.5 ml of 3.8% sodium citrate. Plasma for measurement of sP-selectin, ß-TG, F1+2, D-dimer, fibrinogen, IL-6, TNF-α, and CRP was separated from the blood sample within 15 min of collection and stored at –70°C until analysis.

Flow cytometry analysis. Whole blood staining. Four-color analysis was used for detection of platelets, leukocyte activation, and leukocyte-platelet complexes. Within 5 min of venipuncture, 5 µl of whole blood was incubated in 50 µl phosphate buffered saline (PBS; Unimedic AB, Matfors, Sweden), pH 7.4, containing saturating concentration of phycoerythrin (PE)-conjugated anti-CD11b (clone D12), peridinin chlorophyll protein (PerCP)-conjugated anti-CD61 (clone RUU-PL7F12), allophycocyanin (APC) anti-CD45 (clone 2D1), and fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin (CD62P; clone AC1.2) or anti-PAC-1 (clone PAC-1) or anti-CD64 (clone 32.2). All antibodies were obtained from Becton Dickinson, except CD64, which was kindly provided by Dr. B. Davis (Maine Medical Research Institute, Scarborough, ME, USA). As isotype controls, we used IgM-FITC (Immunotech, Marseille, France), IgG1-FITC/IgG2-PE (Dako Cytomation, Glostrup, Denmark), and Simultest IgG1-FITC/IgG2a-PE, IgG1-PerCP, IgG2-PE (Becton Dickinson). The same batches of monoclonal antibody (mAb) were used for all quantitative measurements. After 30 min incubation with the mAb, in the dark at room temperature, all samples were resuspended in 950 µl of PBS containing 1% paraformaldehyde (Cell fix; Becton Dickinson). The stained blood samples were stored on ice in the dark and analyzed on a flow cytometer within 2 h of fixation.

Acquisition and interpretation. Samples were analyzed with a FACS Calibur flow cytometer (Becton Dickinson), which was calibrated daily with QC Windows, Quantum Molecules of Equivalent Soluble Fluorochrome (Bangs Laboratories, Fishers, IN, USA), and CaliBrite (Becton Dickinson) beads, and with cells as described[19]. The cell calibration standard was represented by peripheral blood lymphocytes stained separately with anti-CD3 mAb conjugated with FITC, PE, PerCP, and APC, then fixed, mixed, and kept for 5 days at 4°C.

Analysis of leukocyte activation and leukocyte-platelet complexes. A previously described method[20] was used with some modification. Briefly, the 4-color analysis included CD45-APC as the FL4 fluorochrome. FSC and SSC signals were set in linear mode, and the fluorescent signals in logarithmic mode. Roughly 10,000 CD45-positive events were collected with a live gate applied on FSC versus FL4 (CD45 as a leukocyte identification marker) cytograms in order to eliminate debris. The analysis was performed with individual gates applied on lymphocytes, monocytes, and neutrophils (at least 500 events in each gate). The staining protocol included FL1 (CD62P, PAC-1, or CD64), FL2 (CD11b), and FL3 (CD61 as a platelet identification marker). CellQuest software (Becton Dickinson) was used for analysis. The FL2 versus FL3 cytograms (CD11b vs CD61) were used to estimate leukocytes that were free or in complexes with platelets. FL1 versus FL3

cytograms (CD64 or PAC-1 vs CD61) were used to measure leukocytes in complexes with platelets or free (CD64). Quadrant markers were applied based on isotype control cytograms. Complexes of lymphocytes, monocytes, or neutrophils with platelets were measured by estimating the percentage of double-positive cells (CD11b+ or CD64+ and CD61+) reported for all lymphocytes, monocytes, or neutrophil populations. Concomitantly, the complexes of lymphocytes, monocytes, or neutrophils with PAC-1+ platelets were evaluated by estimating the percentage of double-positive cells (PAC-1+, CD 61+) reported for all platelets in the respective gate (lymphocytes, monocytes, or neutrophils, respectively).

For measurement of platelet activation, i.e., presence of CD62P and PAC-1 on free platelets, a second acquisition of data was performed with instrument settings specific for platelet analysis.

Quantitative flow cytometry. The geometric mean of fluorescence intensity (MFI) was calculated for all quantitative measurements, as the CellQuest software does not give the median of fluorescence intensity in the quadrant statistics. The MFI of CD11b and CD64 on free leukocytes and of PAC-1 and P-selectin on free platelets was measured and further translated into molecules of equivalent soluble fluorochrome (MESF). The procedure involved the use of Quantum™ 26-FITC and Quantum™ 26-PE (Bangs Laboratories). The software accompanying the beads translates the channel values, which are instrument-specific, into absolute units of fluorochrome molecules bound per cell. Indirectly, this procedure allows the estimation of antibody binding sites. Based on the fluorescence/protein ratio obtained from the manufacturer, the MESF were converted into antibody binding capacity (ABC).

Biochemical markers. sP-selectin was measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Coefficient of variation (CV) was 9.9% at 94 ng/ml and 8.8% at 451 ng/ml. CRP was assessed by immunoturbidimetry using the DiaSys reagent (Diagnostic Systems, Holzheim, Germany) (CV was 8.5% at 20 mg/ml and 6.8% at 42 mg/ml). ESR was analyzed using the Sedimatic 100 (Analysinstrument, Stockholm, Sweden).

IL-6 concentrations were measured by ELISA, using the Quantikine Human IL-6 Immunoassay kit (R&D Systems) (CV 6.4% at 17 pg/ml and 3.3% at 100 pg/ml). TNF-α was determined using the Easia TNF-α kit (Bio-Source Europe, Nivelles, Belgium) (CV 8% at 163 pg/ml and 9.9% at 644 pg/ml). F1+2 was analyzed by ELISA, Enzygnost F1+2 kit (Dade Behring, Naperville, IL, USA) (CV 11.6% at 0.92 nmol/ml). D-dimer was measured by ELISA, Vidas D-Dimer New (bioMerieux, Marcy l'Etoile, France) (CV 5% at 0.5 mg/l fibrinogen equivalent unit). Fibrinogen was assessed by the Claus method using a Fibri-Prest Automate (Diagnostica Stago, Asnieres, France) (CV 2.4% at 2.9 g/l and 3.5% at 0.8 g/l). ß-TG was measured by ELISA using a kit from Asserachrom (Diagnostica Stago) and CV was 11.3%.

Statistical analysis. Data were compared by Wilcoxon matched-pairs rank test. P values < 0.05 were considered significant. Associations were estimated using Pearson's correlation coefficient. The StatView 5 program (Abacus Concepts Inc., Berkeley, CA, USA) was used for statistical calculations.

RESULTS

Platelet activation. The expression of P-selectin and PAC-1 binding before and after MTX or TNF blocker therapy was compared with the healthy controls (Table 1). P-selectin ABC before and after therapy was not significantly changed compared to controls in the MTX-T and TNF-T groups. The ABC of PAC-1 was significantly increased in MTX-T ($p < 0.02$) and TNF-T ($p < 0.005$) groups before therapy. After therapy, the PAC-1 remained significantly elevated above the normal levels in MTX-T ($p < 0.03$) and TNF-T ($p < 0.02$) groups.

sP-selectin levels were also significantly higher before therapy in MTX-T ($p < 0.04$) and TNF-T ($p < 0.02$) groups than in controls (Table 1). TNF blocker significantly reduced sP-selectin ($p < 0.02$), but the levels were still increased compared to controls ($p < 0.02$).

ß-TG concentration was not significantly different in all patients before therapy compared to controls (Table 1). However, TNF blocker significantly diminished ß-TG levels ($p < 0.007$).

Leukocyte activation. The ABC of CD11b and CD64 on monocytes in patients and controls is presented in Table 2. Before therapy, the ABC of CD11b was significantly elevated in MTX-T ($p < 0.04$) and TNF-T ($p < 0.007$) groups compared to controls. The CD11b ABC remained elevated after MTX ($p < 0.007$) or TNF blocker ($p < 0.007$) therapy. A significant increase of CD64 ABC was also found in MTX-T ($p < 0.01$) and TNF-T ($p < 0.003$) groups before therapy compared to controls. After therapy the reduction of CD64 ABC on monocytes was significant only in the MTX-T group ($p < 0.04$). Results presented in Table 3 show the ABC of CD11b and CD64 on neutrophils. The ABC of CD11b on neutrophils was initially slightly increased in the MTX-T group, but became significantly higher compared to controls after therapy ($p < 0.02$). In the TNF-T group, the CD11b ABC on neutrophils was significantly higher both before and after therapy ($p < 0.02$). The CD64 ABC on neutrophils was also significantly higher in all patients before therapy compared to controls ($p < 0.02$). After TNF blocker therapy the levels of CD64 ABC on neutrophils remained significantly increased ($p < 0.007$).

Leukocyte-platelet complexes. The interaction of a component of $\alpha_M ß_2$ integrin (CD11b) and FcγRI (CD64) with platelets in patients compared to controls is illustrated in Figures 1 and 2.

CD11b-positive leukocyte-platelet complexes. Before therapy all patients showed a significantly higher percentage of monocyte-platelet complexes compared to controls ($p < 0.02$; Figure 1). After therapy, there was no significant decrease in percentage of monocyte-platelet complexes in patients. Also, the lymphocyte-platelet and neutrophil-platelet complexes at Day 0 were slightly increased in all patients (Figure 1). After MTX therapy, the percentage of neutrophil-platelet complexes was still increased (Figure 1).

CD64-positive leukocyte-platelet complexes. Compared to controls, the percentage of monocyte-platelet complexes before therapy was elevated in MTX-T ($p < 0.009$) and TNF-T ($p < 0.01$) groups (Figure 2). After therapy with MTX, the percentage of monocyte-platelet complexes was still significantly increased ($p < 0.02$). The increase in the percentage of neutrophil-platelet complexes in all patients before therapy did not achieve significance (Figure 2).

Leukocyte-PAC-1-positive platelet complexes. The interaction of leukocytes with PAC-1-positive platelets is illustrated in Figure 3. Before therapy, the percentage of monocytes

Table 1. Platelet activation markers from controls and patients with RA on Day 0 and after 6 weeks of therapy. Results are median (interquartile range).

	Controls	MTX-T		TNF-T	
		Day 0	Week 6	Day 0	Week 6
P-selectin, ABC	2655 (2403–2973)	2594 (2521–3455)	2725 (2296–3272)	3348 (2678–3864)	3785 (2418–4686)
PAC-1, ABC	452 (387–648)	589* (533–845)	594** (478–775)	763*** (578–882)	746* (531–798)
sP-selectin, ng/ml	27 (18–43)	78† (66–85)	68* (64–86)	89* (79–98)	72*†† (56–82)
ß-TG, IU/ml	26 (20–30)	25 (21–39)	29 (19–46)	29 (23–41)	18# (15–20)

* $p < 0.02$ vs controls, ** $p < 0.03$ vs controls, *** $p < 0.005$ vs controls, † $p < 0.04$ vs controls, †† $p < 0.02$ vs Day 0, # $p < 0.007$ vs Day 0. ABC: antibody binding capacity.

Table 2. Expression of CD11b and CD64 on monocytes from controls and patients with RA on Day 0 and after 6 weeks of therapy. Results are median (interquartile range).

	Controls	MTX-T		TNF-T	
		Day 0	Week 6	Day 0	Week 6
CD11b, ABC	26,152 (23,390–29,205)	30,439* (24,200–39,270)	33,301** (26,668–36,338)	39,883** (28,250–68,133)	32,398** (26,677–55,402)
CD64, ABC	4593 (4276–5300)	5746*** (5576–8434)	5129†† (4578–6779)	6951† (5698–7537)	6079** (5143–6461)

* $p < 0.04$ vs controls, ** $p < 0.007$ vs controls, *** $p < 0.01$ vs controls, † $p < 0.003$ vs controls, †† $p < 0.04$ vs day. ABC: antibody binding capacity.

Table 3. Expression of CD11b and CD64 on neutrophils from controls and Patients with RA on Day 0 and after 6 weeks of therapy. Results are median (interquartile range).

	Controls	MTX-T		TNF-T	
		Day 0	Week 6	Day 0	Week 6
CD11b, ABC	15,303 (12,828–20,165)	18,411 (12,261–22,475)	25,343* (17,440–30,974)	29,449* (15,404–39,992)	27,435* (14,474–38,393)
CD64, ABC	583 (496–632)	638* (587–700)	609 (512–878)	684* (660–1006)	709** (686–846)

* $p < 0.02$ vs controls, ** $p < 0.007$ vs controls. ABC: antibody binding capacity.

in complex with platelets showed significantly elevated levels in the MTX-T ($p < 0.02$) and TNF-T ($p < 0.03$) groups. A significant reduction in the percentage of monocytes in complex with platelets was observed after TNF blocker therapy ($p < 0.02$). Before therapy the percentage of neutrophils in complex with platelets was significantly elevated only in the TNF-T group ($p < 0.006$). After therapy, the percentage of neutrophil-platelet complexes was significantly increased in the MTX-T group ($p < 0.01$) compared to controls. No significant change was observed in the percentage of lymphocyte-platelet complexes before or after therapy in any group (Figure 3). The percentage of monocyte-platelet complexes detected by CD11b, CD64, or PAC-1 at Day 0 was higher in the MTX-T group than in the TNF-T group.

Clinical and inflammation variables. The changes in biochemical inflammatory markers and clinical activity are shown in Table 4. The patients had significantly higher levels of CRP, ESR, IL-6, and TNF-α compared to controls before therapy. The numbers of tender and swollen joints were 18.5 and 15 in the MTX-T group and 20 and 19, respectively, in the TNF-T group. There was a significant decrease in the numbers of tender and swollen joints in all patients after therapy (Table 4). In the TNF-T group the improvement in disease activity was accompanied by significant declines of CRP ($p < 0.009$), ESR ($p < 0.02$), IL-6 ($p < 0.04$), and TNF-α ($p < 0.009$).

Coagulation variables. Significantly higher levels of D-dimer were found before therapy in the MTX-T ($p < 0.007$) and TNF-T ($p < 0.02$) groups then in controls (Table 5). After MTX therapy, D-dimer concentrations decreased significantly ($p < 0.007$). Compared to controls, elevated fibrinogen levels were found in all patients before therapy ($p <$

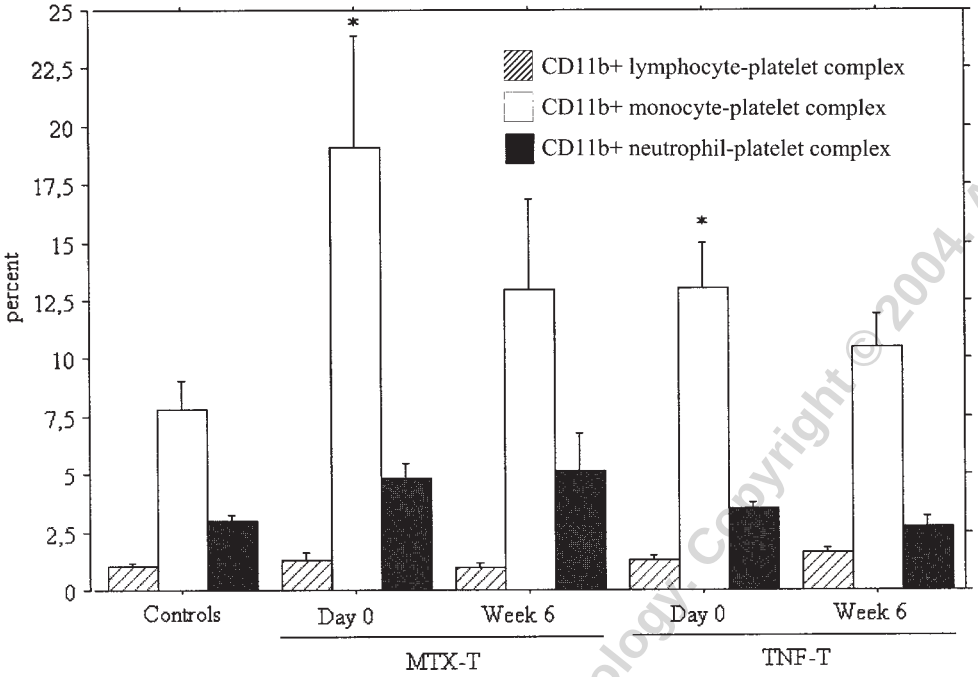

Figure 1. Percentage of CD11b+ lymphocyte-platelet, CD11b+ monocyte-platelet, and CD11b+ neutrophil-platelet complexes from RA patients and controls on Day 0 and after 6 weeks of therapy. Results are mean ± SEM. *p < 0.02 vs controls.

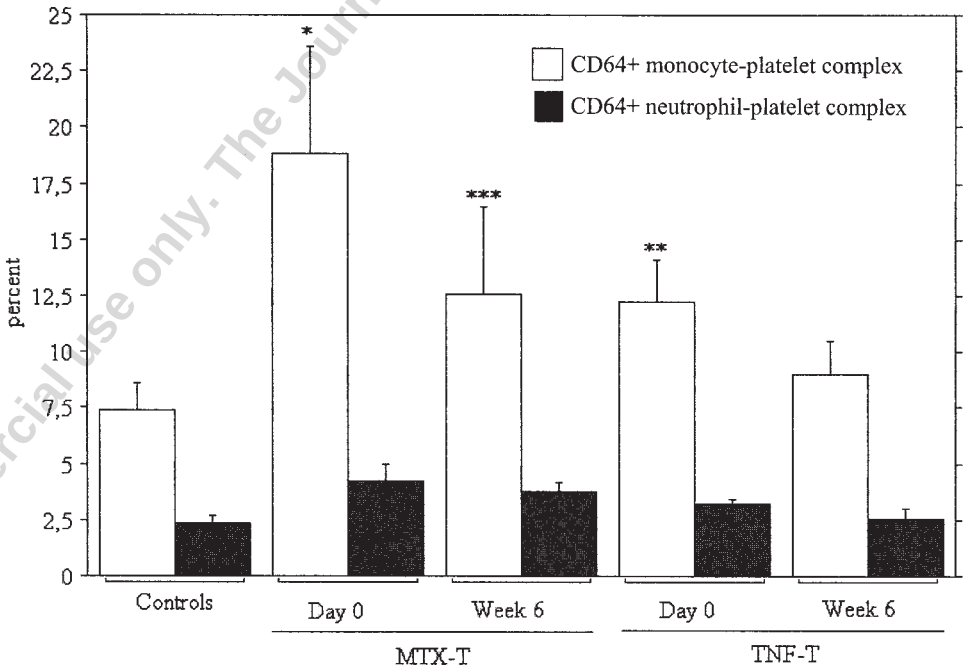

Figure 2. Percentage of CD64+ monocyte-platelet and CD64+ neutrophil-platelet complexes from RA patients and controls on Day 0 and after 6 weeks of therapy. Results are mean ± SEM. *p < 0.009 vs controls, **p < 0.01 vs controls, ***p < 0.02 vs controls.

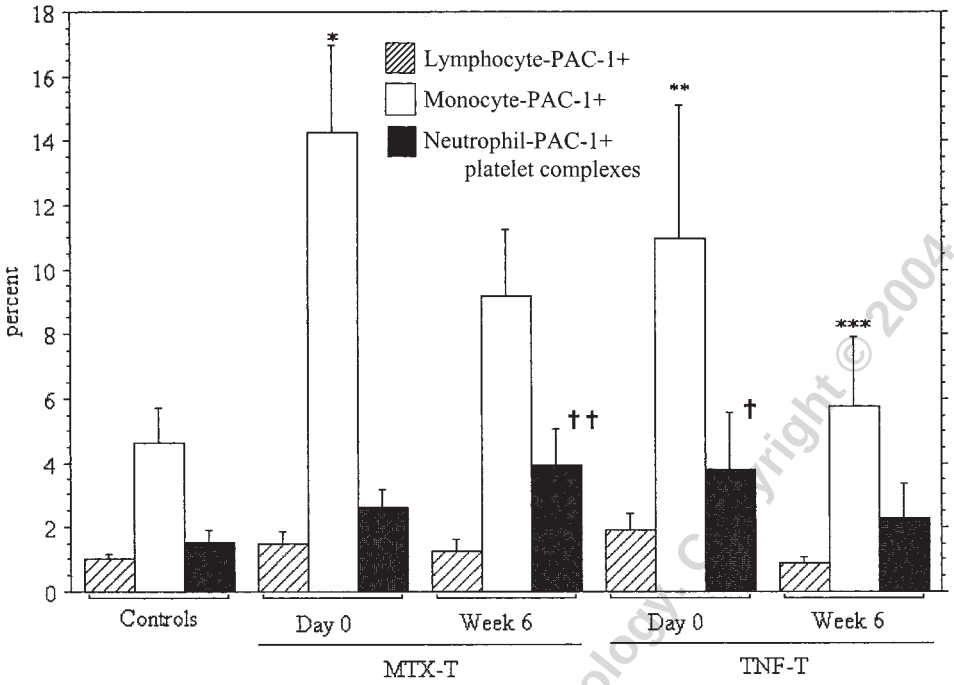

Figure 3. Percentage of lymphocyte-PAC-1+ platelet, monocyte-PAC-1+ platelet, and neutrophil-PAC-1+ platelet complexes from RA patients and controls on Day 0 and after 6 weeks of therapy. Results are mean ± SEM. *p < 0.02 vs controls, **p < 0.03 vs controls, ***p < 0.02 vs Day 0, †p < 0.006 vs controls, ††p < 0.01 vs controls.

Table 4. Clinical and inflammation markers from controls and patients with RA on Day 0 and after 6 weeks of therapy. Results are median (interquartile range).

	Controls	MTX-T		TNF-T	
		Day 0	Week 6	Day 0	Week 6
CRP, mg/l	3 (2.0–5.0)	6.9* (4.3–56)	6.0** (4.0–13)	29*** (12–114)	6.5**† (5.0–13)
ESR, mm/h	5 (4–5)	18* (6–38)	10.5*** (5–23)	43.5*** (34–55)	22**†† (16–29)
IL-6, pg/ml	3.7 (2.1–6.2)	51* (4.9–94.5)	4.6 (1.1–8.6)	58* (20–129)	6.0# (3.9–19.5)
TNF-α, pg/ml	16 (14–19)	40* (32–61)	29 (21–36)	52** (21–130)	18† (9.5–33)
Tender joints	ND	18.5 (17–19)	13† (8–14)	20 (19–21)	11.5## (9–16)
Swollen joints	ND	15 (12–18)	10### (10–12)	19 (17–19)	11## (8–15)

* p < 0.04 vs controls, ** p < 0.02 vs controls, *** p < 0.01 vs controls, † p < 0.009 vs Day 0, †† p < 0.02 vs Day 0, # p < 0.04 vs Day 0, ## p < 0.005 vs Day 0, ### p < 0.007 vs Day 0. ND: not done.

0.02; Table 5). TNF blocker reduced the fibrinogen levels significantly (p < 0.005). F1+2 levels were not significantly different in all patients before therapy compared to controls, but diminished significantly after MTX therapy (p < 0.04; Table 5).

Correlation between leukocyte-platelet activation markers and disease activity before treatment (Day 0). MTX-T group. The CD11b ABC on monocytes correlated with the swollen joint count in this group (r = 0.83, p < 0.003) and CD64 ABC on monocytes correlated with CRP (r = 0.68, p < 0.03) and ESR (r = 0.85, p < 0.002), respectively. CD11b+ monocyte-platelet complexes correlated with CD64+ monocyte-platelet (r = 0.97, p < 0.001) and monocyte-PAC-1+ platelet (r = 0.75, p < 0.009) complexes.

TNF-T group. The percentage of CD11b+ monocyte-platelet complexes correlated with the tender joint count (r = 0.66, p

Table 5. Coagulation variables from controls and patients with RA on Day 0 and after 6 weeks of therapy. Results are median (interquartile range).

	Controls	MTX-T		TNF-T	
		Day 0	Week 6	Day 0	Week 6
D-dimer, mg/l	0.2 (0.2–0.3)	2.2* (1.2–3.9)	0.7† (0.3–1.0)	2.7** (1.2–4.0)	1.2*** (0.5–1.8)
Fibrinogen, g/l	2.6 (2.0–3.4)	4.6** (3.2–6.2)	3.9** (2.9–5.1)	6.1** (5.2–6.4)	3.9**†† (3.8–4.3)
F1 + 2, nmol/l	1.0 (1.8–1.2)	1.4 (1.1–1.6)	0.9# (0.7–1.0)	0.9 (0.7–1.4)	0.8 (0.6–1.1)

* $p < 0.007$ vs controls, ** $p < 0.02$ vs controls, *** $p < 0.04$ vs controls, † $p < 0.007$ vs Day 0, †† $p < 0.005$ vs Day 0, # $p < 0.04$ vs Day 0.

< 0.03). CRP levels were associated with fibrinogen (r = 0.85, p < 0.001) and ESR (r = 0.71, p < 0.01).

Correlation between leukocyte-platelet activation markers, disease activity, coagulation, and fibrinolysis markers after 6 weeks of therapy. MTX-T group. The CD64 ABC on monocytes was associated with CRP (r = 0.68, p < 0.04), ESR (r = 0.73, p < 0.02), and D-dimer (r = 0.84, p < 0.002).

CRP levels correlated with fibrinogen (r = 078, p < 0.009), ESR (r = 0.84, p < 0.001), IL-6 (r = 0.99, p < 0.001), D-dimer (r = 0.79, p < 0.003), and CD11b+ monocyte-platelet (r = 0.89, p < 0.002), CD64+ monocyte-platelet (r = 0.89, p < 0.0001) and CD11b+ neutrophil-platelet (r = 0.92, p < 0.0001) complexes. An association was also found between IL-6 and CD11b+ monocyte-platelet complexes (r = 0.95, p < 0.008), CD64+ monocyte-platelet complexes (r = 0.96, p < 0.006), and fibrinogen (r = 0.88, p < 0.01).

D-dimer levels also correlated with fibrinogen (r = 0.84, p < 0.002), ESR (r = 0.94, p < 0.001), IL-6 (r = 0.95, p < 0.001), and CD11b+ monocyte-platelet (r = 0.75, p < 0.04), CD64+ monocyte-platelet (r = 0.75, p < 0.008) and CD11b+ neutrophil-platelet (r = 0.77, p < 0.005) complexes.

TNF-T group. CRP levels were associated with ESR (r = 0.91, p < 0.0001), IL-6 (r = 0.75, p < 0.009), D-dimer (r = 0.96, p < 0.0001), and fibrinogen (r = 0.75, p < 0.009). D-dimer correlated with fibrinogen (r = 0.67, p < 0.03), ESR (r = 0.83, p < 0.001), and IL-6 (r = 0.78, p < 0.005) levels.

DISCUSSION

We applied a standardized 4-color flow cytometry analysis for quantitative measurement of activation markers on platelets (P-selectin, PAC-1) and leukocytes (CD11b, CD64) in patients with RA and healthy controls before and after therapy with MTX or TNF blocker. Concomitantly, the leukocyte-platelet interaction was estimated as leukocyte-platelet complexes. To our knowledge this is the first report to quantify PAC-1 binding and study the influence of MTX or TNF blocker treatment on platelet activation and leukocyte-platelet interaction in RA. The platelet activation was estimated by quantitative measurements of P-selectin (α granule membrane component) expression and PAC-1 binding, determination of sP-selectin concentration, and ß-TG (α granule matrix component) release in plasma. No difference was observed in P-selectin expression in RA patients compared to controls or in the effects of MTX or TNF blocker. Plasma concentrations of sP-selectin were significantly increased in RA patients before therapy, in accord with the findings of other investigators[21-23]. TNF blocker therapy was more effective than MTX alone in decreasing sP-selectin levels. In agreement with Veale, *et al*[21] we found no correlation between sP-selectin and disease activity. The source and function of sP-selectin in RA patients remain to be established. Although sP-selectin may also reflect the release of P-selectin from activated endothelial cells, most sP-selectin, it has been suggested, is derived from platelets[24-26]. It has been proposed that sP-selectin induces procoagulant activity associated with an increased risk of vascular and thrombotic disease[27]. The binding of PAC-1 to platelets was significantly increased before therapy in all patients. No therapeutic effect of MTX or TNF blocker on PAC-1 binding was observed, suggesting persistent platelet (GPIIb/IIIa) activation.

As in other studies, we found high expression of CD11b on monocytes in RA patients[28,29]. Neutrophil expression of CD11b was also elevated in patients compared to controls. MTX therapy resulted in increased expression of CD11b on monocytes and neutrophils, while the effect of TNF blocker therapy was weak on CD11b expression. Our study revealed significantly higher monocyte CD64 expression in patients than in controls, indicating that RA is associated with a higher state/degree of monocyte activation[28,29]. The correlation between CD64 expression on monocytes and CRP and ESR indicates an upregulation of CD64 due to the inflammatory response, possibly by FcγR-mediated phagocytosis and FcγR-triggered generation of reactive oxygen intermediates. MTX therapy was more effective than TNF blocker in reduction of CD64 expression on monocytes. We found increased CD64 expression on neutrophils in patients compared to controls. However, no correlations with CRP or ESR were detected. Some studies have shown no increased CD64 expression on the circulating neutrophils of patients with RA[30,31], in contrast with the findings of Allen, *et al*[32]. However, increased CD64 neutrophil expres-

sion has been described in synovial fluid of patients with RA[30,31,33].

Increased circulating levels of monocyte (CD11b+ or CD64+)-platelet complexes, neutrophil (CD11b+ or CD64+)-platelet complexes, and lymphocyte (CD11b+)-platelet complexes were found in patients compared to controls. After treatment, the levels of monocyte (CD11b+ or CD64+)-platelet complexes, neutrophil (CD64+)-platelet complexes in the MTX-T and TNF-T groups, and neutrophil (CD11b+)-platelet complexes in the MTX-T group remained increased compared to controls, suggesting a state of persistent leukocyte-platelet activation in RA. The positive correlation observed between CRP and IL-6 levels and the monocyte (CD11b+ or CD64+)-platelet complexes and neutrophil (CD11b+)-platelet complexes could indicate an association between inflammation and activation of the hemostatic system. Circulating levels of monocyte-PAC-1+ platelet complexes were significantly elevated in all patients before therapy and remained elevated after therapy, suggesting sustained platelet activation. No relationship was found between these complexes and concentrations of CRP, ESR, or cytokine.

Elevated levels of fibrinogen were observed in patients compared to controls, and were most likely related to the degree of inflammation. Large amounts of fibrin have been reported to be deposited in the joints of patients with RA[34]. It has also been reported that RA patients have elevated levels of D-dimers, possibly due to fibrinolytic degradation of fibrin[35,36]. The correlation between D-dimer and fibrinogen with CRP and ESR supports the relationship between alterations of hemostatic factors and the inflammatory activity in RA. Treatment with MTX or TNF blocker seems to lead to decreased synthesis of plasma fibrinogen as part of the diminished inflammatory reaction. Provided that the fibrinolytic process is part of the fibrinogen catabolic pathway, the reduced fibrinogen synthesis leads to a decrease of D-dimer formation.

McEntegart, *et al* found that patients with RA have an increased prevalence of angina pectoris and possibly of stroke[37]. Both these syndromes are related to arteriosclerosis and thrombotic events. Our findings of increased platelet activation [PAC-1 binding, sP-selectin, monocyte (CD11b+, CD64+)-platelet complexes, and monocyte-PAC-1+ platelet complexes] and increased levels of D-dimer may suggest an increased activation of the hemostatic system and consequently an increased risk of thrombotic events in rheumatoid patients. Concentrations of F1+2, a marker of coagulation, were not different from the controls, indicating no increased generation of thrombin.

Our results indicate increased platelet activation (PAC-1 binding, sP-selectin) and leukocyte activation (CD11b, CD64), and increased formation of monocyte (CD11b+, CD64+)-platelet complexes and monocyte-PAC-1+ platelet complexes in patients with RA, suggesting a status of simultaneous activation of the immune and the hemostatic systems. Therapy with a TNF blocker was more effective than MTX alone in decreasing platelet activation (monocyte-PAC-1+ platelet complexes, sP-selectin), but the effect was minimal on leukocyte activation (CD11b, CD64), neutrophil-PAC-1+ platelet complexes, and monocyte and neutrophil (CD11b+, CD64+)-platelet complexes. The fibrinogen receptor activation of GPIIb/IIIa, assessed as PAC-1 binding, was not inhibited by TNF blocker or MTX therapy. Further studies are needed to elucidate the interaction between immune and hemostatic systems in patients with RA.

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