Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis


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Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis

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ABSTRACT

Objectives Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterised by synovitis and joint destruction. The pathogenesis of RA is not clear, but is considered to be an immune-mediated inflammatory disorder, in which the complement system plays an important role. Although cell-derived microparticles (MPs) have been associated with inflammation and complement activation, it is unknown whether MPs are either cause or consequence. Therefore, we investigated whether circulating MPs differ between patients with very early as yet untreated arthritis and healthy controls, and whether intensive anti-inflammatory treatment of such patients affects circulating MPs.

Methods Patients with RA (n=24) and controls (n=15) were included. Nine patients with RA were re-evaluated after 8 weeks of intensive treatment with a combination of drugs (‘COmBination therapy in Rheumatoid Arthritis’ (COBRA) scheme). Disease activity was measured by erythrocyte sedimentation rate (ESR), C reactive protein (CRP) and Disease Activity Score for 28 joints (DAS28). Flow cytometry was used to study MPs and exposure of complement activator molecules and complement components.

Results At baseline, concentrations of MPs exposing C1q, CRP or serum amyloid-P (SAP) were all significantly elevated in patients with early RA compared to controls (p=0.003, p=0.002 and p=0.003, respectively). Upon treatment, DAS28 score, ESR and CRP levels significantly decreased (p=0.008, p=0.008 and p=0.012), but the concentrations of circulating MPs and MPs exposing complement components or activator molecules were unaffected.

Conclusion Circulating MPs exposing complement components or activator molecules are elevated in early RA. Since a strong anti-inflammatory therapy suppressed inflammation in patients with early RA but not levels of circulating MPs, it is unlikely that inflammation is the main underlying cause of MP release in these patients.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with a complex pathogenesis, characterised by synovitis leading to cartilage, tendon and joint destruction.1 2 Although the pathogenesis of RA is not clear, it is considered to be an immune-mediated inflammatory disorder, in which the complement system plays an important role.

Recently, cell-derived microparticles (MPs), which are small membrane vesicles released from blood cells or endothelial cells upon activation or during apoptosis, were shown to be associated with complement activation, inflammation and coagulation in various diseases, including inflammatory diseases.3–7 Although inflammation causes release of MPs and in turn MPs may induce or enhance inflammation, it remains unknown whether circulating MPs merely reflect ongoing inflammation or whether MPs actually contribute to the disease development.

In vitro studies and animal models indicate that inflammatory mediators such as tumour necrosis factor α (TNFα) and interleukin (IL)1 trigger MP release.8–10 Several other observations, however, suggest a more active role for MPs in development of rheumatoid inflammatory activity. Leucocyte-derived MPs, present in synovial fluid of patients with RA, expose tissue factor and trigger coagulation,11 and induce the production and release of chemokines and cytokines by fibroblast-like synoviocytes, which in turn may further contribute to synovial inflammation and angiogenesis.12

Circulating platelet-derived MPs (PMPs) were reported to be elevated in patients with RA, compared to controls, and these PMPs were associated with disease activity as measured by the Disease Activity Score in 28 joints (DAS28).13 Finally, synovial MPs may also be involved in complement activation in patients with RA, since we recently demonstrated the presence of bound complement components C1q, C3 and C4 as well as complement activator molecules on circulating MPs from patients with RA, further supporting their role in complement activation.4

To determine whether circulating MP numbers are associated with inflammatory activity in patients with RA, we compared MPs in patients with very early as yet untreated arthritis and healthy controls. Additionally, we determined the effects of changes in disease activity upon intense anti-inflammatory therapy with the ‘COmBination therapy in Rheumatoid Arthritis’ (COBRA) strategy14 on MP numbers and composition.

METHODS

Patients

Consecutive untreated patients with RA (n=24) were included and venous blood was collected at baseline in the fasting state. Of these patients, nine were enrolled in a trial addressing the effects of tight control and intensified COBRA combination treatment in early RA and were treated with COBRA treatment comprising sulfasalazine, methotrexate and high-dose step-down prednisolone at 60 mg/day (week 1), 40 mg/day (week 2),
30 mg/day (week 3), 20 mg/day (week 4), 15 mg/day (week 5),
10 mg/day (week 6) and 7.5 mg/day thereafter.\textsuperscript{14} Criteria
for inclusion in that study were active disease defined by a DAS28
score >3.2. Of these nine patients, an additional fasting blood
sample was collected after 8 weeks of treatment. Furthermore,
fasting blood was collected from healthy age-matched controls
(n=15). All patients fulfilled the criteria of the American College
of Rheumatology (ACR) for RA.\textsuperscript{15} All participants gave written
informed consent and the study protocol was approved by the
Institutional Ethics Committee of the Slotervaart Hospital, Jan
van Breemen Institute and BovenIJ Hospital.

Collection of blood samples
Participants were asked to refrain from beverages other than
water (particularly no caffeine-containing beverages or alco-
hol), smoking, medication and meals from midnight prior to
the testing day. Blood was collected from the antecubital vein
in tubes containing 0.5 ml of 3.2% sodium citrate (BD, San Jose,
California, USA). Cells were removed by centrifugation (20 min
at 1550 g and 20°C) within 10 min after collection. Aliquots of
cell-free plasma (250 μl) were snap frozen in liquid nitrogen for
at least 15 min and stored at $-80°C$.\textsuperscript{16}

Isolation of MPs
MPs were isolated from plasma aliquots (250 μl) after thawing
on melting ice by centrifugation (30 min at 18,890 g and 20°C).
After centrifugation, MP-free supernatant (225 μl) was removed.
The remaining MP pellet was washed with 225 μl phosphate-
buffered saline (PBS) containing (0.32% w/v) trisodium citrate
(pH 7.4). After centrifugation, the supernatant was removed and
the MP pellet was resuspended in PBS-citrate (75 μl).

Labelling of MPs
Aliquots of MPs (5 μl) were diluted in 35 μl of PBS containing 2.5
mmol/litre CaCl\textsubscript{2} (PBS/Ca, pH 7.4). Subsequently, 5 μl allophycocyanin (APC)-labelled annexin V (CalTag Laboratories, Carlsbad,
California, USA) was added and combined with either fluores-
cine isothiocyanate (FITC)-labelled CD61 (DakoCytomation,
Glostrup, Denmark) plus phycoerythrin (PE)-labelled CD62p
(P-selectin) or CD63 (glycoprotein 55; both antibodies from
Immunotech, Fullerton, California, USA), or FITC-labelled
CD144 (Alexis, San Diego, California, USA) plus E-selectin
(CD62e-PE; Ancell, Bayport, Minnesota, USA). For appropri-
ate settings of fluorescence thresholds, MPs were incubated with
isotype-matched control antibodies, that is, PE-labelled
IgG\textsubscript{1} and/or FITC-labelled IgG\textsubscript{1} (BD), or FITC-labelled Ig (IQP,
Groningen, The Netherlands). MPs were labelled for 15 min at
room temperature, and labelling was stopped by addition of
PBS/calcium (900 μl) to each tube. Samples were analysed for
1 min by fluorescence-activated cell sorting (FACS) on a FACS
Calibur device (BD) and data were analysed using CellQuest Pro
(V.4.0.2; BD).\textsuperscript{17}

Alternatively, MPs (5 μl aliquots) were incubated for 30 min at
room temperature with anti-C1q, anti-C3-15, anti-C reactive
protein (CRP), GC4, anti-serum amyloid P (SAP)-14, anti-IgM,
anti-IgG (gift from Sanquin, Amsterdam, The Netherlands) or
isotype-matched control antibodies IgG\textsubscript{1} and IgG\textsubscript{2a} (Pharmica,
Montlingen, Switzerland) in a final volume of 50 μl of PBS con-
taining 2.5 mmol/litre CaCl\textsubscript{2} (PBS/Ca, pH 7.4). After labelling,
MPs were washed with PBS/calcium (200 μl). Subsequently,
PE-labelled F(ab\textsuperscript{‘})\textsubscript{2} and APC-labelled annexin V were added and
the mixtures were incubated for 30 min at room temperature.
For setting of fluorescence thresholds, MPs were incubated
with isotype-matched control antibodies, that is, PE-labelled
IgG\textsubscript{1} and/or FITC-labelled IgG\textsubscript{1} (BD), or FITC-labelled Ig (IQP).
Finally, PBS/calcium (400 μl) was added to each tube and sam-
pies were analysed for 1 min on a FACS Calibur device. Data
were analysed using CellQuest Pro.\textsuperscript{6} All antibodies and control
antibodies used were tested and titrated using purified cells and
MPs before use.

Identification and characterisation of MPs
MPs were defined according to size (forward scatter), side scatter
and binding of annexin V, a protein that binds with high affi
nity and specificity to phosphatidylserine, as described previously.\textsuperscript{18} It
should be mentioned, that the percentage of MPs binding
annexin V increases by centrifugation and freeze-thawing.
Under these conditions binding of annexin should be considered
as a marker to identify MPs rather than reflecting the exposure
of negatively charged phospholipids such as phosphatidylserine.
The within-run coefficient of variation (CV) is 8% and the day-
to-day CV is 13%. The presence of bound complement com-
ponents (C1q, C3 and C4) as well as bound adapter molecules
(CRP, SAP, IgM and IgG) was studied using flow cytometry as
described previously.\textsuperscript{4}

Statistical analysis
Data were analysed with SPSS for Windows V.16.0 (SPSS,
Chicago, Illinois, USA). According to their distribution, the vari-
ous parameters are expressed as mean (±SD) or median (inter-
quartile range). Data with a non-Gaussian distribution was log
transformed for analysis if possible. To compare the groups,
Student t tests or Mann–Whitney U tests were used when
appropriate. Furthermore, correlations between variables were
analysed by using Pearson correlation or Spearman rho tests.
Univariate linear regression analyses were performed on log-
transformed data to investigate the influence of possible con-
founders (ie, sex, smoking status, systolic blood pressure and
body mass index (BMI) on the results). The Wilcoxon signed-
rank test was used to investigate the differences in values at
baseline and at 8 weeks in the prospectively followed subgroup
of patients (n=9). p Values less than 0.05 were considered statis-
tically significant.

RESULTS
Characteristics and inflammatory measures
Baseline demographic and clinical characteristics of the patients
with RA are summarised in table 1. The majority of patients
with RA were IgM-rheumatoid factor and/or anti-citrullinated
protein antibody (ACPA) positive. Their DAS28 scores (mean
5.2) reflect patients with a high disease activity. Erythrocyte sed-
imentation rate (ESR) and CRP levels were significantly elevated
in patients compared to controls. The patient group comprised
fewer women and had higher systolic blood pressure than the
controls. The nine patients prospectively followed had similar
ages, BMI levels, systolic blood pressure index and body mass
index (BMI) on the results). The Wilcoxon signed-
rank test was used to investigate the differences in values at
baseline and at 8 weeks in the prospectively followed subgroup
of patients (n=9). p Values less than 0.05 were considered statis-
tically significant.

Elevated concentrations of MPs exposing complement
components or activator molecules in early RA
The total number of MPs did not differ between patients and
controls (table 2). In patients, the number of MPs exposing C1q,
CRP and SAP were significantly elevated compared to con-
trols (table 2 and figure 1). These results remained unchanged
after adjusting for possible confounders (data not shown).
At baseline, ESR and CRP significantly correlated with MPs exposing C1q, CRP and SAP (for ESR: r=0.37, p=0.02; r=0.54, p<0.001 and r=0.46, p=0.003, respectively and for CRP: r=0.39, p=0.02; r=0.52, p=0.001 and r=0.36, p=0.02, respectively), confirming the association between ongoing inflammation and circulating MPs.

Intense inflammatory suppression does not alter MP composition

Upon treatment with intense anti-inflammatory therapy, DAS28, ESR and CRP values decreased significantly (table 3), but the concentrations of total circulating MPs and MPs exposing complement components or activator molecules were unaffected (table 3, figure 2). Numbers of MPs exposing C1q or CRP were still significantly elevated in the patients with RA after treatment compared to controls (data not shown). At 8 weeks we did not find correlations between DAS28 and CRP with total MP numbers or MPs exposing complement components or activator molecules.

DISCUSSION

This study demonstrates that MPs exposing complement components (C1q) or activator molecules (CRP or SAP) are elevated in early active RA. Although a strong anti-inflammatory therapy using a combination of disease-modifying anti-rheumatic drugs (DMARDs) combined with high-dose prednisolone (under the COBRA scheme) strongly suppressed inflammatory activity, circulating MPs were unaffected. Our present data may suggest that inflammation is not the underlying cause of MP generation in these patients.

Imaging studies have shown that synovitis is still apparent in the majority of patients with RA that are clinically in remission, indicating subclinical ongoing inflammation. The present findings suggest that MPs may be one of the factors that are actively involved in this sustained inflammation in RA. We cannot exclude, however, that a delay exists between normalisation of systemic inflammation and circulating MPs or their

### Table 1 Baseline characteristics of the patients with rheumatoid arthritis (RA) (n=24) and healthy controls (n=15)

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=15)</th>
<th>Patients (n=24)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>49±11</td>
<td>51±11</td>
<td>0.91</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>13 (87)</td>
<td>14 (58)</td>
<td>0.02</td>
</tr>
<tr>
<td>RF positive, n (%)</td>
<td>NA</td>
<td>16 (67)</td>
<td>NA</td>
</tr>
<tr>
<td>Anti-CCP n (%)</td>
<td>NA</td>
<td>17 (71)</td>
<td>NA</td>
</tr>
<tr>
<td>DAS28</td>
<td>NA</td>
<td>5.2±1.3</td>
<td>NA</td>
</tr>
<tr>
<td>ESR</td>
<td>5 (3–8)</td>
<td>33 (15–48)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>1 (1–2)</td>
<td>13 (3–37)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NSAID use, n (%)</td>
<td>NA</td>
<td>17 (77%)</td>
<td>NA</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>7</td>
<td>30</td>
<td>0.13</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>119±7.8</td>
<td>131±22.8</td>
<td>0.03</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>79±5.9</td>
<td>79±10.8</td>
<td>0.93</td>
</tr>
<tr>
<td>BMI</td>
<td>23.4±2.0</td>
<td>25.1±4.3</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD or median (interquartile range), as applicable.

### Table 2 Complement component and activator molecule exposing MPs in patients and controls

<table>
<thead>
<tr>
<th>MPs and complement components</th>
<th>Controls (n=15)</th>
<th>Patients (n=24)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MPs</td>
<td>103.6 (64.5–129.9)</td>
<td>108.9 (70.4–185.0)</td>
<td>0.27</td>
</tr>
<tr>
<td>C1q</td>
<td>4.7 (1.7–8.8)</td>
<td>9.4 (5.5–14.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>C4</td>
<td>11.6 (7.0–15.8)</td>
<td>17.8 (7.6–25.0)</td>
<td>0.35</td>
</tr>
<tr>
<td>C3</td>
<td>11.3 (7.7–15.0)</td>
<td>9.1 (2.5–19.5)</td>
<td>0.34</td>
</tr>
<tr>
<td>CRP</td>
<td>3.0 (1.3–4.6)</td>
<td>7.3 (2.5–19.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>SAP</td>
<td>47.9 (22.9–59.5)</td>
<td>95.3 (62.0–155.6)</td>
<td>0.003</td>
</tr>
<tr>
<td>IgM</td>
<td>18.4 (11.5–34.2)</td>
<td>24.4 (14.4–42.3)</td>
<td>0.43</td>
</tr>
<tr>
<td>IgG</td>
<td>3.6 (1.0–6.6)</td>
<td>3.3 (2.0–6.5)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Values are presented as median (IQR). p Values were calculated using the Mann–Whitney U test.

CRP, C reactive protein; MPs, microparticles (numbers ×10⁴/ml); SAP, serum amyloid-P.
Table 3  Inflammatory markers, complement component and activator molecule exposing MPs in a subgroup of patients treated with anti-inflammatory drugs at baseline and after 8 weeks

<table>
<thead>
<tr>
<th>Disease scores and MPs</th>
<th>Baseline (n=9)</th>
<th>Week 8</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28</td>
<td>5.2 ± 0.7</td>
<td>2.2 ± 1.2</td>
<td>0.008</td>
</tr>
<tr>
<td>ESR</td>
<td>45 (17–62)</td>
<td>12 (6–27)</td>
<td>0.008</td>
</tr>
<tr>
<td>CRP</td>
<td>19 (9–69)</td>
<td>4 (1–8)</td>
<td>0.008</td>
</tr>
<tr>
<td>Total MPs</td>
<td>167 (73–239)</td>
<td>106.7 (36.9–266)</td>
<td>0.401</td>
</tr>
<tr>
<td>MPs + C1q</td>
<td>14.4 (8.4–24.4)</td>
<td>13.4 (4.2–29.8)</td>
<td>0.089</td>
</tr>
<tr>
<td>MPs + C4</td>
<td>22.2 (5.9–27.9)</td>
<td>19.1 (7.6–58.3)</td>
<td>1.0</td>
</tr>
<tr>
<td>MPs + C3</td>
<td>10.3 (4.1–21.4)</td>
<td>12.3 (1.9–37.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>MPs + CRP</td>
<td>7.9 (3.4–31.2)</td>
<td>7.1 (2.9–28.1)</td>
<td>0.575</td>
</tr>
<tr>
<td>MPs + SAP</td>
<td>108.2 (53.4–172.8)</td>
<td>109.3 (29.7–214)</td>
<td>0.779</td>
</tr>
<tr>
<td>MPs + IgM</td>
<td>31.6 (13.7–41.4)</td>
<td>34.1 (15.1–63.1)</td>
<td>0.401</td>
</tr>
<tr>
<td>MPs + IgG</td>
<td>2.3 (0.3–6.0)</td>
<td>1.2 (0.0–3.2)</td>
<td>0.674</td>
</tr>
</tbody>
</table>

Values are median (IQR). p Values were calculated using the Wilcoxon signed-rank test. After total MPs, the other categories refer to the number of MPs exposing a certain complement component or activator molecules.

CRP C reactive protein; DAS28, disease activity score of 28 joints; ESR, erythrocyte sedimentation rate; MPs, microparticles (numbers ×10⁴/ml); SAP, serum amyloid-P.

composition. Alternatively, subpopulations of circulating MPs may be affected by normalisation of inflammation rather than the total population of MPs, as assessed in the present study. The biological relevance of MPs or subpopulations thereof to the pathology of early active RA may be questioned given the fact that inflammation and disease activity were both efficiently suppressed.

Recently, MPs have emerged as a new proinflammatory mediator. In fact, they are thought to amplify or disseminate inflammation. MPs are thought to trigger inflammation by several processes such as activation of endothelial cells and leucocytes, triggering production and release of chemokines and cytokines and by activating the complement cascade, which is thought to play a key role in the pathogenesis of RA.

By contrast, inflammation may trigger MP formation. For instance, in vitro studies showed that MPs are released from cells incubated with TNFα or IL1 and a study in mice showed that the number of PMPs in plasma markedly increased upon injection with TNFα. Data from the present study, however, implicate that MPs remain associated with complement activation in early RA despite aggressive anti-inflammatory therapy.

We cannot answer the question yet as to whether these MPs really have proinflammatory properties and thus actively contribute to complement activation, or whether they merely reflect ongoing complement activation. In the 1980s, Sims and coworkers demonstrated that cells were protected from complement-induced lysis by the release of complement complex-enriched MPs. Thus, the presence of elevated concentrations of complement-enriched MPs in early RA may also be a reflection of ongoing and uncontrolled activation of the complement system.

Our main finding that powerful inhibition of inflammation did reduce disease activity but did not disturb the association between circulating MPs and complement activation, suggests that MPs and complement contribute to the development of and/or the chronic character of inflammatory diseases such as RA.

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Competing interests  None.

Ethics approval  This study was conducted with the approval of the Institutional Ethics Committee of the Slotervaart Hospital, Jan van Breemen Institute and BovenIJ Hospital.

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Extended report


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