Synovial fluid from patients with rheumatoid arthritis inhibits neutrophil apoptosis: role of adenosine and proinflammatory cytokines

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Abstract

Objective. In synovial fluid (SF) from patients with rheumatoid arthritis (RA), neutrophils are exposed to proinflammatory mediators endowed with either anti-apoptotic or pro-apoptotic properties. We investigated neutrophil apoptosis in the presence of SF from 11 RA patients.

Methods. SF was obtained from affected knees of 11 patients with RA. Human neutrophil apoptosis was evaluated by light microscopic examination and flow-cytometric analysis of annexin V binding. Immune complex-induced neutrophil activation was evaluated as superoxide anion production. Adenosine levels in SF were detected by chromatographic analysis and cytokine levels were studied by enzyme-linked immunosorbent assay.

Results. Spontaneous and immune complex-triggered neutrophil apoptosis was reduced by SF from eight out of 11 patients. Immune complex-induced neutrophil activation was unaffected by SF. The cytokines tested had no role in promoting the anti-apoptotic activity of SF. On the contrary, the anti-apoptotic activity of SF was found to depend on the presence of adenosine. Adenosine levels detected in the various samples of SF correlated significantly with the anti-apoptotic activity of the fluids and with the number of apoptotic neutrophils detected in the articular exudate.

Conclusion. The microenvironment of rheumatoid SF is a proinflammatory milieu responsible for the in loco persistence of activated and long-surviving neutrophils. Adenosine plays a crucial role in this phenomenon, which is related to anti-apoptotic activity.

Key words: Neutrophils, Rheumatoid arthritis, Apoptosis, Adenosine, GM-CSF, TNF-α.

Rheumatoid arthritis (RA) can be considered a prototype for the diseases characterized by neutrophilic inflammation. In fact, huge amounts of neutrophils appear in the synovial fluid of RA patients, where the neutrophil count can be more than $5 \times 10^4$ per mm$^3$ [1]. Neutrophils have also been detected at the pannus–cartilage junction at sites of erosion [2, 3], where they release potent effectors of cartilage destruction, such as serine and metalloproteases [4, 5]. In agreement with these observations, activated monocytes and fibroblastoid cells secreting interleukin (IL) 8, a major neutrophil chemoattractant, have been detected not only in the lining layer of the synovial tissue but also at the pannus–cartilage junction [6]. Thus, neutrophils have been considered to be among the cells with great potential to inflict damage within the RA joint [7]. RA fluids can also contain large quantities of immune complexes, and the deposition of immune complexes has been considered to be a major determinant of neutrophil-mediated articular tissue injury [8]. Indeed, it is well known that neutrophil Fcγ receptor (FcγR) cross-linking by immune complexes leads to full cell activation [9]. Consequently, immune complex-activated neutrophils discharge large quantities of toxic products, overwhelming the antioxidant and antiprotease shields of synovial fluid [10, 11]. In turn, this leads to the development of tissue injury which, when relevant and/or persistent, is responsible for the irreversible anatomical destruction of the normal tissue architecture, with consequent articular dysfunction [12, 13]. More recently, it has been shown that neutrophils engaged in FcγR-dependent effector functions, such as immune
complex phagocytosis, undergo accelerated apoptosis [14, 15]. This is a crucial topic. It is generally accepted that neutrophil apoptosis is essential in the prevention of excessive tissue damage by decreasing the tissue load of neutrophils, neutrophil function and the histotoxic burden of neutrophils [16]. In contrast to necrotic cells, apoptotic neutrophils are promptly recognized and engulfed by macrophages without stimulating the proinflammatory activities of phagocytosing cells [17, 18]. Therefore, from a teleological point of view, immune complex-mediated apoptosis may be a mechanism to accelerate the disposal of exhausted neutrophils, thereby favouring the resolution of neutrophilic inflammation [16]. Nevertheless, proinflammatory cytokines capable of prolonging neutrophil survival are also detectable in RA synovial fluids [19–22]. Thus, the fate of neutrophils at sites of inflammation, where these cells are likely to be exposed to both anti- and pro-apoptotic influences, needs to be clarified. To investigate this issue, we studied the survival of neutrophils in the presence of articular fluids from RA joints before and after immune complex activation.

Materials and methods

Medium and reagents

RPMI 1640 with 25 mM HEPES (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal calf serum (FCS) (ICN Biomedicals, Milan, Italy) was used as the incubation medium. Dulbecco’s phosphate-buffered saline (PBS) and Hanks’ balanced salt solution (HBSS) were from Irvine Scientific. Heparin was obtained from Roche (Milan, Italy). Ficoll-Hypaque was purchased from Seromed (Berlin, Germany). Fluorescein diacetate, ethidium bromide, propidium iodide, human albumin, rabbit anti-human albumin immunoglobulin (Ig) G, ferri-cytochrome c, superoxide dismutase, N-ethylmaleimide and 3,7-dimethyl-1-proparglyanthine (DMPX) were from Sigma-Aldrich (Milan, Italy). The Annexin V-FITC (FITC = fluorescein isothiocyanate) kit was purchased from Boehringer Ingelheim (Heidelberg, Germany). Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and human recombinant IL-6 were from Genzyme (Cambridge, MA, USA). Human recombinant IL-2, human recombinant 72-amino acid IL-8, human recombinant IL-15 and human recombinant tumour necrosis factor-α (TNF-α) were from Biosource International (Camarillo, CA, USA). Other reagent-grade compounds were used as obtained from commercial suppliers.

Patients

Synovial fluid was obtained after informed consent from the affected knees of 11 patients with RA diagnosed according to established criteria [23]. The mean duration of disease was 3 ± 1 yr and the mean age was 51 ± 8 yr. Patients (seven females, four males) were taking the following medications: non-steroidal anti-inflammatory drugs (NSAIDs; n = 9), methotrexate 7.5 mg/week (n = 2), low-dose prednisone 2.5 mg/day (n = 4), auranofin 6 mg/day (n = 1). No local intra-articular corticosteroid injections had been made within 5 weeks before the collection of synovial fluid samples and no patients had been treated with TNF-α blockers or leflunomide. Synovial fluid was also obtained from three patients affected with osteoarthritis (two without anti-inflammatory treatment, one taking an NSAID).

Preparation of synovial fluid samples

Immediately after synovial fluid collection, cells were counted with a haemocytometer and cytospins were prepared for the morphological assessment of apoptosis, as described below. Then synovial fluid samples were centrifuged at 1000 g for 15 min. Finally, the supernatants were removed aseptically and stored at −20°C in aliquots.

Preparation of immune complexes

Immune complexes were prepared by incubating human albumin and rabbit anti-human albumin IgG at equivalence, which was determined, on the basis of quantitative precipitin curves, at the molar ratio 1:5, as described previously [15]. Briefly, albumin and rabbit anti-human albumin IgG were incubated for 2 h at 37°C and thereafter overnight at 4°C. Subsequently, immune complexes were washed three times (500 g for 10 min), resuspended in cold PBS and stored at 4°C. The total protein in the precipitates was determined with the Lowry assay [24].

Neutrophil isolation and culture

Heparinized (heparin 10 U/ml) venous blood was obtained from healthy volunteers after informed consent. Neutrophils were isolated by dextran sedimentation, subsequent centrifugation on a density gradient and removal of contaminating erythrocytes by hypotonic lysis [15]. The resulting neutrophils were washed three times with incubation medium, and the final cell suspensions (2 × 10^6/ml) always contained 97% or more viable cells. Neutrophils were then incubated in tissue culture tubes (17 × 100 mm; Falcon, Becton Dickinson, Oxnard, CA, USA) at 37°C in a 5% CO2 atmosphere (final volume 0.5 ml). Experiments were carried out in the absence or presence of immune complexes (25 μg/ml), GM-CSF (10 ng/ml), IL-2 (1000 U/ml), IL-6 (1000 U/ml), IL-8 (10 ng/ml), IL-15 (10 ng/ml) or TNF-α (10 ng/ml). The concentrations of immune complexes, chemokines and cytokines were chosen on the basis of preliminary dose–response experiments (data not shown). At appropriate time points, cells were harvested and counted on a haemocytometer before subsequent assays.

Neutrophil membrane integrity assay

Neutrophil viability, measured as the integrity of the cell membrane, was assessed according to Dankberg and Perdisky [25] as described previously [15]. Briefly, cells (4 × 10^6/100 μl) harvested from culture tubes were mixed with 50 μl of staining solution (2 μg/ml fluorescein diacetate, 4 μg/ml ethidium bromide in HBSS) and...
incubated for 10 min at room temperature. Thereafter, a drop of cell suspension was placed on a slide, sealed with a coverslip and analysed under ultraviolet light with dark-field illumination. Neutrophils with an intact membrane (i.e. viable cells) appeared as green fluorescent cells, whereas neutrophils with a damaged and ethidium bromide-permeable membrane (i.e. necrotic cells) displayed a fluorescent red nucleus.

**Light microscopic assessment of neutrophil apoptosis**
Cytocentrifuged cell preparations were fixed and stained with May–Grünwald–Giemsa. Thereafter, two independent observers read cytopreparations blindly by oil-immersion light microscopic examination of at least 500 cells/slide (magnification 1000 × ). Cells showing apoptotic morphology were identified according to the typical criteria of cell shrinking, nuclear condensation and fragmentation, plasma membrane ruffling and blebbing [26], as described previously [15].

**Immunofluorescence flow cytometry of annexin V-FITC binding**
Immunofluorescence analysis of annexin V binding was performed with the Annexin V-FITC Kit according to the manufacturer's instructions with minor changes, as described previously [15]. Briefly, cells were washed and resuspended in 100 µl isotonic binding buffer. Then annexin V-FITC (3 µl) was added and, after incubation (15 min), cells were washed and resuspended in ice-cold PBS supplemented with 3% FCS and 0.1% sodium azide. Flow cytometry analysis was performed on an EPICS XL flow cytometer (Coulter, Hialeah, FL, USA). Living granulocytes were gated on the basis of physical properties (forward vs side light scatter) and at least 2000 living cells were analysed for each sample. Flow cytometer settings for the gating and analysis of neutrophils were adjusted in conformity with results from preliminary experiments performed on CD15-stained purified neutrophils.

**Superoxide anion release assay**
The release of superoxide anion was studied by using a modification of the method of Babior et al. [27] as described previously [28]. Briefly, neutrophils (5 × 10⁵) were incubated (20 min, 37°C, final volume 0.5 ml) with 80 µM ferricytochrome c in the absence or presence of 300 U/ml superoxide dismutase (SOD). The reactions were then stopped by adding 2 ml of ice-cold 1 mM N-ethyl maleimide and superoxide production was determined in the supernatants from the optical density at 550 nm (OD₅₅₀) of samples without SOD minus the OD₅₅₀ of samples with SOD, using an extinction coefficient of 2.1 × 10³ M⁻¹ cm⁻¹.

**Determination of GM-CSF and TNF-α levels in synovial fluid samples**
Synovial fluid samples were filtered on pore size 0.8 µm (Millipore S.p.A., Vimodrone, Italy) and treated with 150 U/ml hyaluronidase (Sigma). After incubation (20 min, 37°C), the concentrations of GM-CSF and TNF-α were measured by immunoassays from Medgenix Diagnostics (Fleurus, Belgium) (GM-CSF) and BioSource (TNF-α).

**Determination of adenosine levels in synovial fluid samples**
Adenosine levels in synovial fluids were determined chromatographically, as detailed elsewhere [29]. The chromatographic analyses were performed with a Perkin-Elmer (Norwalk, PA, USA) Model Series 4 liquid chromatograph, a Rheodyne 7125 (Berkeley, CA, USA) injector valve with a 10 µl loop, and a Perkin-Elmer model LC35 diode-array UV detector. Retention times, peak areas and UV spectra were recorded with a Perkin-Elmer LCI-100 integrator. A 250 × 4.6 mm internal diameter stainless-steel Hibal column pre-packed with 5 µm RP-18 phase was used (Hypersil, Runcorn, UK); the column was protected with a LiChroCart 4–4 guard cartridge system (Merck, Darmstadt, Germany). The mobile phase consisted of acetonitrile–0.01 M monobasic sodium phosphate (5:95 v/v), and it was delivered with a high-pressure solvent-mixing valve. The flow rate was 1.0 ml/min. Detection was performed at 260 nm. All separations were carried out at room temperature. Calibration curves were constructed by plotting the peak area of standard working aqueous solutions of adenosine (γ) against concentration (c) using linear regression analysis [29]. Unknown concentrations of adenosine were quantified by relating the respective peak area to the regression line.

**Determination of immune complex levels in synovial fluid samples**
The concentrations of immune complexes in synovial fluids were measured with an immunoassay from Dia.Metra, Segrate-Milano, Italy, according to the manufacturer’s instructions.

**Immunodepletion of cytokines in synovial fluid**
Synovial fluid samples were immunodepleted of GM-CSF and TNF-α singly and of GM-CSF and TNF-α together, by adding to the synovial fluid the appropriate neutralizing polyclonal antibodies (60 µg/ml; Genzyme, Cambridge, MA, USA). The antibody concentration was chosen in order to achieve >90% neutralization of cytokine activity, on the basis of neutralization assays performed by the manufacturer. After incubation (overnight, 4°C), synovial fluids were ultracentrifuged (10 000 g, 45 min). After immunodepletion, the cytokines were undetectable in all samples, as measured by the above-mentioned immunoassays. Therefore, supernatants were tested immediately in the apoptosis assay, as described above.

**Statistical analysis**
Data were expressed as mean ± S.D. Analyses were performed with GraphPad Instat and GraphPad Prism (GraphPad Software, San Diego, CA, USA).
Differences were determined by one-way analysis of variance with Bonferroni multiple comparison tests. Correlations between pairs of variables were assessed by the Pearson method. Differences were accepted as significant when \( P < 0.05 \).

Results

**Effect of RA synovial fluids on neutrophil apoptosis**

The effects of RA synovial fluids on neutrophil apoptosis were studied in two experimental conditions: (i) spontaneous apoptosis evaluated after 18 h *in vitro* incubation and (ii) apoptosis of immune complex-activated neutrophils after 12 h of incubation. The time-points were chosen on the basis of our previous observations [15] to allow detection of inhibition as well as acceleration of the apoptotic process. Neutrophils from normal donors were incubated in the absence or presence of 50% synovial fluid for the appropriate period of time, then apoptosis was determined morphologically. As shown in Fig. 1, neutrophil apoptosis was significantly reduced by co-incubation with eight of 11 synovial fluids obtained from inflamed joints of distinct RA patients. This was observed in both spontaneous (Fig. 1A) and immune complex-stimulated (Fig. 1C) neutrophil apoptosis. Moreover, as shown in Table 1, time-course experiments performed with selected synovial fluid samples from patients 1, 5 and 7.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Medium</th>
<th>Medium + SF</th>
<th>IC</th>
<th>IC + SF</th>
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<tr>
<td>3</td>
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<td>2.7 ± 1.2</td>
<td>4.8 ± 0.1</td>
<td>2.7 ± 0.3</td>
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<tr>
<td>6</td>
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<td>6.7 ± 3.5</td>
<td>19.4 ± 4.9</td>
<td>11.3 ± 3.7</td>
</tr>
<tr>
<td>12</td>
<td>24.0 ± 5.6</td>
<td>15.0 ± 5.0</td>
<td>56.1 ± 8.1</td>
<td>32.5 ± 9.8</td>
</tr>
<tr>
<td>18</td>
<td>54.6 ± 11.1</td>
<td>34.0 ± 13.9</td>
<td>74.6 ± 5.8</td>
<td>51.3 ± 11.5</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of cells showing apoptosis. Apoptosis was evaluated morphologically on cytopreparations stained with May–Grünewald–Giemsa. IC, 25 μg/ml. Data are mean ± s.d. of three experiments performed with SF samples from patients 1, 5 and 7.

[Fig. 1. Effect of RA synovial fluids on neutrophil apoptosis. (A) Spontaneous neutrophil apoptosis was assessed after 18 h of incubation in the absence (open bars) or presence (filled bars) of 50% synovial fluids from 11 patients with RA. Apoptosis was evaluated morphologically on cytopreparations stained with May–Grünewald–Giemsa. One-way analysis of variance, \( P < 0.0001 \). Bonferroni multiple comparison test for patients 1 (n = 9), 4 (n = 7), 5 (n = 8), 7 (n = 4), 8 (n = 4), 9 (n = 4), 10 (n = 4) and 11 (n = 4), apoptosis in the absence vs presence of synovial fluid, \( P < 0.001 \); patients 2 (n = 5), 3 (n = 6) and 6 (n = 3), apoptosis in the absence vs presence of synovial fluid, \( P > 0.05 \). Data are mean and s.d. (B) Morphological features of neutrophils incubated in culture medium in the absence (left) and presence (right) of synovial fluid. Cells showing apoptotic characteristics, such as condensed nuclei and apoptotic bodies, are indicated with arrows. (C) Neutrophil apoptosis triggered by 25 μg/ml immune complexes was assessed after 12 h of incubation in the absence (open bars) or presence (filled bars) of 50% synovial fluids from 11 patients with RA. One-way analysis of variance, \( P < 0.0001 \). Bonferroni multiple comparison test for patients 1 (n = 4), 4 (n = 4), 5 (n = 4), 7 (n = 4), 8 (n = 4), 9 (n = 4), 10 (n = 4) and 11 (n = 4), apoptosis in the absence vs presence of synovial fluid, \( P < 0.001 \); patients 2 (n = 5), 3 (n = 4) and 6 (n = 4), apoptosis in the absence vs presence of synovial fluid, \( P > 0.05 \). Data are mean and s.d. (D) Morphological features of neutrophils incubated with immune complexes in the absence (left) or presence (right) of synovial fluid. Cells showing apoptotic characteristics are indicated with arrows.]
samples showed an evident anti-apoptotic action from the 6 h time point of neutrophil ageing. The inhibitory activity of the eight synovial fluids was dose-dependent in both systems (not shown). In all cases, neutrophil viability, assessed as membrane integrity, was more than 97%. Similar results were obtained in selected experiments using autologous neutrophils purified from peripheral blood of RA patients (data not shown).

**Effect of proinflammatory cytokines on neutrophil apoptosis**

Neutrophil apoptosis can be modulated by several soluble ligands, including cytokines detectable at sites of inflammation [19–22]. In an attempt to identify the factor(s) responsible for the anti-apoptotic activity of synovial fluids, the effects of six cytokines on spontaneous and immune complex-induced neutrophil apoptosis were studied. Neutrophils were incubated in the absence or presence of GM-CSF (10 ng/ml), IL-2 (1000 U/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), IL-15 (10 ng/ml) or TNF-α (10 ng/ml). The concentration of each cytokine was chosen on the basis of preliminary dose–response experiments (not shown). After the appropriate incubation time, apoptosis was determined morphologically and confirmed, using a flow cytometer, by the annexin V binding assay. As shown in Fig. 2, a significant reduction in the percentage of spontaneous apoptosis was observed in the presence of GM-CSF, IL-6, IL-15 or TNF-α, but not of IL-2 or IL-8. Furthermore, only GM-CSF and TNF-α displayed anti-apoptotic properties with respect to immune complex-dependent neutrophil apoptosis (Fig. 3). We did not observe anti-apoptotic activity even when the inactive ligands were used at 100-fold higher concentration (data not shown). Taking into account the fact that some reports about the effects of TNF-α on neutrophil apoptosis are conflicting, some indicating that this cytokine has pro-apoptotic activity and others that it has an anti-apoptotic role [30–34], we performed a series of time-course and dose-response experiments. As reported in Table 2, TNF-α exerted a slight pro-apoptotic effect at earlier time-points (at 3 and 6 h for spontaneous apoptosis and at 3 h for immune complex-induced apoptosis), whereas potent anti-apoptotic activities were observed in the presence of GM-CSF, IL-6, IL-15 or TNF-α.
activity was observed at subsequent time-points. Finally, the capacity of TNF-α to retard neutrophil ageing was confirmed in dose–response experiments, as shown in Table 3.

Inhibition of neutrophil apoptosis by synovial fluids does not depend on GM-CSF and TNF-α

The results reported above suggest that the inhibition of spontaneous and immune complex-stimulated neutrophil apoptosis by synovial fluids may depend on the presence of GM-CSF and/or TNF-α in the articular effusions. Consequently, we determined the synovial fluid concentrations of GM-CSF and TNF-α. Both cytokines were detected in the 11 fluids examined, at concentrations ranging from 0.5 to 28.1 pg/ml for TNF-α and from 0.9 to 414.0 pg/ml for GM-CSF. Nevertheless, no relationship was found between the GM-CSF and TNF-α concentrations measured in each synovial fluid studied and the levels of neutrophil apoptosis detected in the presence of the same synovial fluid (Fig. 4). Accordingly, immunodepletion of GM-CSF and TNF-α had no effect on the anti-apoptotic activity of synovial fluids (data not shown).

Inhibition of neutrophil apoptosis by synovial fluids depends on adenosine

Two recent reports suggest that neutrophil apoptosis can be down-modulated by adenosine [35, 36], a ubiquitous autacoid endowed with immune-modulatory properties towards several cell types, including neutrophils [37, 38]. Evidence for the anti-apoptotic activity of adenosine was obtained by using adenosine analogues and selective adenosine receptor agonists [35, 36]; at present there is no direct evidence for neutrophilic anti-apoptotic activity of adenosine. Thus, a series of experiments was planned to investigate the possible role of adenosine in the observed activity of RA synovial fluids on neutrophil apoptosis. We found that adenosine was capable of slowing the rate of spontaneous apoptosis of neutrophils incubated for 18 h [mean (s.d.) inhibition of neutrophil apoptosis in the presence of 10 μM adenosine, 18.1 ± 17.8%, n = 5; inhibition of neutrophil apoptosis in the presence of 100 μM adenosine, 31.1 ± 17.7, n = 5]. We then verified the anti-apoptotic activity of synovial fluids morphologically and by flow cytometry (annexin V binding assay) in the absence or presence of 0.25 U/ml adenosine deaminase, an enzyme capable of removing efficiently any adenosine that is present in the samples. The capacity of each synovial fluid to slow the apoptotic process was almost completely abolished by the treatment with adenosine deaminase. This was observed for both spontaneous apoptosis (Fig. 5) and immune complex-induced apoptosis (Fig. 6). Similar results were obtained by using DMPX, an antagonist of the adenosine A2 receptor (data not shown). No effect was observed when adenosine deaminase was added to neutrophils in the absence of synovial fluid, ruling out the direct pro-apoptotic activity of the enzyme (data not shown). Consequently, adenosine levels in synovial fluids were determined chromatographically. In eight samples, adenosine was detected at concentrations ranging from 18.7 to 52.4 μM. After the addition of adenosine deaminase, the adenosine signal disappeared (not shown). On the contrary, in the remaining synovial fluids (n = 3)

**Table 3.** Dose-related effects of recombinant TNF-α on spontaneous and immune complex (IC)-induced neutrophil apoptosis

<table>
<thead>
<tr>
<th>TNF-α (ng/ml)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
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<tr>
<td>Medium only</td>
<td>45.3 ± 6.2</td>
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<td>29.7 ± 2.1</td>
<td>22.7 ± 4.2</td>
<td>17.6 ± 2.7</td>
</tr>
<tr>
<td>Medium + IC</td>
<td>64.5 ± 6.2</td>
<td>56.2 ± 7.1</td>
<td>47.8 ± 6.9</td>
<td>40.2 ± 5.3</td>
<td>32.6 ± 6.9</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of cells showing apoptosis. Apoptosis was evaluated morphologically on cyto preparations stained with May–Grünewald–Giemsa at 18 h. Data are mean ± s.d. of two experiments.

**Fig. 4.** Relationship between the concentrations of TNF-α (A) or GM-CSF (B) in 11 rheumatoid synovial fluids and the anti-apoptotic activity of the same samples. The means of two determinations of cytokine concentrations in synovial fluid were plotted against the levels of spontaneous apoptosis of normal neutrophils in the presence of 50% synovial fluid, expressed as the mean of three to nine experiments.
adenosine was undetectable, but a readily detectable peak was found after the addition of 2.6 mM adenosine (not shown). Consistent with these findings, the adenosine levels detected in each synovial fluid correlated significantly with their anti-apoptotic activity towards normal neutrophils (r² = 0.8818, P < 0.001) (Fig. 7A) and the number of apoptotic neutrophils detected in the same sample (r² = 0.7017, P < 0.005) (Fig. 7B). On the contrary, no correlation was found between adenosine level and immune complex concentrations measured in each synovial fluid sample by immunoassay (data not shown). Finally, the anti-apoptotic properties of three pauci-inflammatory synovial fluids from different patients affected by osteoarthritis were studied. As shown in Table 4, neutrophil ageing was unaffected by co-incubation with synovial fluids. Consistent with this, adenosine was undetectable in all three samples.

**Effect of RA synovial fluids on neutrophil activation by immune complexes**

Immune complexes are well-known activators of neutrophil oxidative metabolism. On the other hand, adenosine is currently considered a natural anti-inflammatory compound, capable of down-regulating neutrophil activation [38]. Therefore, we evaluated the capacity of synovial fluids to modulate the neutrophil activation triggered by immune complexes. As shown in Fig. 8, synovial fluids were found to be incapable of interfering with neutrophil activation, evaluated as superoxide anion production.

**Discussion**

In the present study, we show that (i) neutrophil survival is significantly prolonged in the presence of inflammatory synovial fluids from patients affected by RA; (ii) RA synovial fluids are also capable of slowing the rate of apoptosis of neutrophils engaged in FcγR-dependent effector functions, i.e. immune complex phagocytosis; and (iii) the exposure of immune complex-triggered neutrophils to synovial fluid does not impair the capacity of these cells to mount an efficient functional response, measured as superoxide anion production. In other words, we provide the first evidence that the
activation-induced acceleration of neutrophil apoptosis can be overwhelmed and countered by anti-apoptotic influences present in RA synovial fluid, which in turn does not affect the capacity of neutrophils to undergo full activation. Taking into account the observation that apoptosis of neutrophils limits their histotoxic potential by favouring removal of these cells from inflamed tissue [16], we propose that the microenvironment of RA synovial fluid is a proinflammatory milieu that is responsible, at least in part, for the persistence of activated and long-surviving neutrophils in the inflamed RA joint.

In 1995, Bell et al. showed that inflammatory synovial fluid contains unidentified factors capable of promoting neutrophil apoptosis [39]. We have no explanation for these contrasting conclusions. Like Bell et al., we studied synovial fluid from patients taking different medications and no correlation was found among the different groups of patients, rendering unlikely the possibility that the differences between the studies can be related to drug-mediated effects. On the other hand, our findings are in agreement with recent reports showing retardation of the spontaneous apoptosis of neutrophils during pathological conditions characterized by neutrophilic inflammation, i.e. acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS) and ulcerative colitis [40–43]. In particular, it has been shown that biological fluids from ARDS and SIRS patients delay neutrophil apoptosis through the anti-apoptotic activity of selected cytokines, such as GM-CSF and TNF-α [40–42]. Exploring this possibility, we found that, among the cytokines investigated, only recombinant GM-CSF and TNF-α are capable of

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**Table 4. Anti-apoptotic activity and adenosine content of pauci-inflammatory synovial fluid**

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<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
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<tr>
<td>Inhibition of spontaneous apoptosis (%)</td>
<td>5.0 ± 7.5</td>
<td>−2.5 ± 9.0</td>
<td>3.2 ± 7.7</td>
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<tr>
<td>Inhibition of IC-induced apoptosis (%)</td>
<td>6.4 ± 11.1</td>
<td>−1.2 ± 6.8</td>
<td>1.6 ± 6.9</td>
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<tr>
<td>Adenosine concentration</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
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</table>

Results are expressed as percentage of cells showing apoptosis. Apoptosis was evaluated morphologically on cytopreparations stained with May–Grünwald–Giemsa at 18 h. Data are mean ± s.d. of three experiments.

IC, immune complex; N.D., not detectable.

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**Fig. 7.** Relationship between the concentration of adenosine in 11 rheumatoid synovial fluids and the anti-apoptotic activity of the same samples. The means of two determinations of cytokine concentrations in synovial fluid are plotted (A) against the levels of spontaneous apoptosis of normal neutrophils in the presence of 50% synovial fluid (mean of three to nine experiments), or (B) against the number/ml of apoptotic neutrophils determined in each sample immediately after arthrocentesis.

**Fig. 8.** Effect of synovial fluids on immune complex (IC)-triggered superoxide anion production by normal neutrophils. Production of superoxide anions (O₂⁻) by neutrophils incubated in the absence (open bars) or presence of 25 μg/ml immune complexes (filled bars) and in the absence (SF−) or presence of 50% synovial fluid (SF+). Data are mean and s.d. of 11 experiments. Production of O₂⁻ in the absence (open bars) vs presence of IC (filled bars), P < 0.001; immune complex-mediated O₂⁻ production in the absence vs presence of synovial fluid, P > 0.05. PMN, polymorphonuclear neutrophils.
inhibiting spontaneous as well as immune complex-mediated apoptosis of normal neutrophils in vitro. Although Salamone et al. [34] showed a profound increase in neutrophil apoptosis after exposure to TNF-α, our results are in accord with previous studies indicating anti-apoptotic activity for this cytokine and, in particular, with data from Murray et al. [31] showing early accelerated apoptosis only in a subpopulation of neutrophils. These contrasting data are the subject of intense scientific discussion, but we did not investigate this issue further, taking into account that inhibition of neutrophil apoptosis by synovial fluids does not depend on TNF-α. Indeed, although we detected TNF-α as well as GM-CSF in RA synovial fluids, we were unable to demonstrate that the anti-apoptotic properties of synovial fluids depend on the presence of these cytokines. Conversely, we provide evidence that the synovial fluid-induced modulation of neutrophil survival is mediated by its adenosine content. This is suggested by the following findings: (i) reagent adenosine is capable of inhibiting neutrophil apoptosis in vitro; (ii) adenosine concentrations strictly correlate with the anti-apoptotic activity of synovial fluid towards normal neutrophils; (iii) adenosine concentrations strictly correlate with the number of apoptotic neutrophils detected in synovial fluids; and (iv) the anti-apoptotic activity of synovial fluids is completely abrogated by adenosine deaminase-mediated depletion of adenosine content. Thus, the ex vivo neutrophilic anti-apoptotic activity of adenosine demonstrated in the present work confirms and extends previous observations obtained in vitro [35, 36].

Adenosine is an endogenous nucleoside released by a variety of normal, stimulated and injured cell types. It is a normal constituent of blood, where it has been detected at concentrations ranging from 20 to 300 nm [44, 45], well below the concentrations we observed in RA synovial fluids. This is not a surprising finding in view of the short plasma half-life of adenosine [46], which is due to its rapid uptake by blood cells, primarily erythrocytes [47]. On the other hand, it is conceivable that, in the extravascular compartment, i.e. in the absence of red cells, the rate of adenosine uptake could be slower and consequently the concentration of the autacoid could be higher. Although few data regarding extravascular adenosine levels are available, in accord with the present findings, adenosine has been measured in alveolar and pericardial fluids at concentrations ranging from to 1 to 200 μM [48, 49].

Adenosine is considered a major modulator of the immune system, endowed with anti-inflammatory properties by acting on different target cells, including neutrophils [37, 38]. Using an ex vivo model of a typical neutrophilic inflammatory disease, i.e. RA, we outline a different scenario. We show that, in the majority of RA synovial fluids examined, all of which were characterized by active neutrophil inflammation, adenosine was detectable at concentrations capable of inhibiting apoptosis of neutrophils, which in turn were fully susceptible to the induction of activation. This suggests that adenosine is likely to display in vivo weak or negligible capacity to inhibit the activation of the neutrophils recruited in the inflamed joint. This is consistent with previous findings showing that adenosine is a poor down-modulator of oxidant production by neutrophils triggered with immune complexes [50]. On the other hand, the presence of soluble factors endowed with neutrophil-priming properties, i.e. GM-CSF and TNF-α, may contribute to the lack of inhibitory activity of adenosine-rich synovial fluids. Accordingly, neutrophils with an activated phenotype are detectable in synovial fluids of RA patients [51], where they are responsible for the inactivation of intra-articular proteinase inhibitors [11] and contribute to the appearance of cartilage/bone erosions [5].

Methotrexate, one of the most widely used anti-rheumatic drugs in the treatment of RA, augments the extracellular release of adenosine, which in turn may contribute to the anti-inflammatory activity of the drug [52]. In particular, several pieces of experimental evidence hint that this drug may exert even neutrophil-targeted anti-inflammatory activities. Indeed, Cronstein et al. [53] found that adenosine released from endothelial cells treated with methotrexate inhibits efficiently the adhesion of activated neutrophils. Furthermore, oral administration of low doses of methotrexate inhibits murine leucocyte recruitment at the site of inflammation by an adenosine-dependent mechanism [54]. Finally, treatment with methotrexate inhibits migration and articular recruitment of neutrophils in patients with RA [55]. Hence, as far as neutrophils are concerned, we propose that the anti-inflammatory activity of methotrexate is probably due to the inhibition of neutrophil recruitment at sites of inflammation, as evidenced by these reports [53–55], without affecting the susceptibility to activation of the recruited cells, as suggested by the present results. On the contrary, the anti-apoptotic activity of adenosine-containing synovial fluids shown in the present work may offer further possible clues about the incomplete or delayed therapeutic responses observed during treatment with methotrexate. From a broader point of view, the variable concentrations of adenosine in RA synovial fluids found in the present work might explain, on the basis of the neutrophil anti-apoptotic activity of the autacoid, the different intensities of synovial neutrophilic inflammation and the possibly different therapeutic responses in different RA patients, as reviewed recently [56].

Due to the relatively small number of synovial fluid samples studied, our results do not offer proven explanations for the differences observed between adenosine levels (and the related anti-apoptotic activity) in 11 samples of RA synovial fluid. Nevertheless, some observations can be made. (i) Although various anti-rheumatic drugs, including methotrexate and salicylate [52, 57], have been reported to affect adenosine release, under our experimental conditions no correspondence was found between drug treatment and adenosine levels in the corresponding synovial fluids. In particular,
neutrophils are exposed to the multiple microenvironmental influences to which neutrophilic inflammation should take into account. (ii) It has been reported that TNF-α is capable of inhibiting adenosine release from some types of cell, including adipocytes [58]. Furthermore, adenosine is capable of inhibiting TNF-α production from activated neutrophils [59]. Nevertheless, no correlation was found between TNF-α and adenosine levels in synovial fluids. (iii) None of the three pauci-inflammatory synovial fluids exerted anti-apoptotic activity and accordingly adenosine could not be detected in them. Nevertheless, much more investigation is needed in order to conclude that the adenosine effects we observed are related to the degree of articular inflammation.

The identification of rational targets for the pharmacological manipulation of neutrophil apoptosis is currently under intensive investigation [60–62]. Our results suggest that the design of therapeutic strategies to increase the rate of neutrophil apoptosis during neutrophilic inflammation should take into account the multiple microenvironmental influences to which neutrophils are exposed in vivo.

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References

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