Extracellular cytochrome c, a mitochondrial apoptosis-related protein, induces arthritis

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Objectives. The aim of the study was to assess the role of extracellular cytochrome c as an inducer of joint inflammation and to examine its levels in sera and synovial fluids of rheumatoid arthritis (RA) patients.

Methods. Mice were injected intra-articularly with different doses of cytochrome c and joints were evaluated histopathologically and immunohistochemically 3 and 10 days later. In addition, mouse spleen cells were stimulated with different concentrations of cytochrome c, followed by assessment of NF-κB activation and cytokine production. Sera and synovial fluid from RA patients and sera from healthy individuals were assessed with respect to cytochrome c levels by an enzyme-linked immunoassay technique.

Results. Histopathological signs of arthritis were evident in 75% of animals following intra-articular injection of cytochrome c. Synovitis was characterized by influx of Mac-1$^+$ cells. *In vivo* depletion of neutrophils and monocytes led to abrogation of arthritis. Stimulation of mouse spleen cells *in vitro* with cytochrome c resulted in activation of NF-κB and release of proinflammatory cytokines and chemokines. Cytochrome c levels in RA patients' sera were significantly lower than in healthy controls. Further, cytochrome c levels in synovial fluid were significantly lower than in corresponding blood samples.

Conclusions. Our findings demonstrate that extracellular cytochrome c displays direct proinflammatory properties mediated by activation of NF-κB and causing neutrophil and monocyte triggered inflammation. We hypothesize that decreased levels of cytochrome c in RA patients reflect consumption of this molecule in the synovial tissue, decreasing apoptosis and shifting the balance towards inflammation.

KEY WORDS: Cytochrome c, Arthritis, Inflammation, NF-κB, Cytokine, Chemokine.

Cytochrome c, a 15-kDa water-soluble mitochondrial haemoprotein, normally resides between the inner and outer mitochondrial membrane. The amino acid sequence of somatic cytochrome c is evolutionarily conserved among species. Indeed, mouse and rat cytochrome c are identical and exhibit 91% homology with the human counterpart [1–3].

Recently cytochrome c came into focus as one of the key signalling molecules of programmed cell death—apoptosis. It has been shown to translocate from mitochondria to the cytosol, where it interacts with apoptotic protease to activate factor-1 together with ATP/dATP and a procaspase 9. This efflux of cytochrome c is a critical event in the activation of intracellular signalling; it results in a cascade of caspase activation and leads to apoptotic cell death [4, 5].

Cytochrome c is an essential component of the respiratory chain of the cell, being responsible for mitochondrial electron transfer from the cytochrome c reductase complex to the cytochrome c oxidase complex [6]. Interruption of this electron transport, which occurs when cytochrome c is depleted from mitochondria, results in the generation of free oxygen radicals and decreased production of ATP [7].

Moreover, cytochrome c can be released into culture fluids from apoptotic as well as from necrotic cells in an intact form [8, 9] and may serve as a marker of cell death *in vivo* in patients with leukaemic malignancies [10] and during myocardial infarction [11–13]. The exact molecular mechanisms responsible for the translocation of cytochrome c from mitochondria during apoptosis are still not clear.

Rheumatoid arthritis (RA) is a systemic autoimmune disease associated with high morbidity and increased mortality. The hallmark of RA is inflammation of the synovial joints, resulting in hyperplasia of synovial fibroblasts and infiltration of macrophages, lymphocytes and plasma cells, which further leads to the destruction of bone and cartilage. Recent findings indicate that the inadequate apoptosis of these inflammatory cells in the RA joint contributes to the pathogenesis of disease [14].

The role of cytochrome c in the pathogenesis of RA has not been studied. Our aim was to analyse the potential proinflammatory role of cytochrome c in a mouse model of arthritis. Our results indicate that (i) cytochrome c triggers synovitis in a healthy host, (ii) this inflammation is mediated by neutrophils, and (iii) cytochrome c can be readily found in the joints of RA patients.

Materials and methods

Patients

Synovial fluid and blood samples were collected from 32 patients (mean age 62 ± 13 yr, mean disease duration 14 ± 9 yr) who met the American College of Rheumatology criteria for RA and attended the Department of Rheumatology, Sahlgrenska University

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Hospital, Göteborg. Synovial fluid was aseptically aspirated and immediately transferred into sodium citrate solution. Blood samples from the same patients were simultaneously obtained from cubital vein into the sodium citrate containing tubes. Blood samples from 16 healthy individuals were used as a control group. Collected blood and synovial fluid samples were centrifuged at 2000 *g* for 10 min, aliquoted, and stored at −20°C until used. The clinical investigation was approved by Ethical Committee of Göteborg University.

The levels of cytochrome *c* in sera and synovial fluid were detected using a standard enzyme-linked immunosorbent assay (ELISA) kit from MedSystems Diagnostics (Vienna, Austria).

Mice

Female NMRI and BALB/c mice, 6–8 weeks old, were purchased from B&K Universal (Stockholm, Sweden). The mice were bred and housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg, Sweden. They were kept under standard conditions of temperature and light, and fed laboratory chow and water *ad libitum*. The study was approved by the Ethical Committee of Göteborg University and the requirements of National Board for Laboratory Animals were followed.

Reagents

Cytochrome *c* from rat heart, displaying 100% homology to corresponding mouse protein, was purchased from Sigma (St Louis, MO, USA), diluted in appropriate concentrations in sterile phosphate-buffered saline (PBS) and tested for lipopolysaccharide (LPS). The total LPS content in the cytochrome *c* solutions was 0.18, 2.3 and 25 pg/μg of protein. The amount of LPS per mouse joint was accordingly 0.18, 2.3 and 25 pg in mice receiving 1, 10 and 100 μg cytochrome *c* respectively. In our previous study, we showed that an intra-articular (i.a.) injection of up to 2 ng of LPS per mouse joint did not induce arthritis [15].

LPS from *Escherichia coli* serotype 055 : B5 and parthenolide were purchased from Sigma.

Injection protocol

Following inhalation (isoflurane) anaesthesia, mice were injected i.a. into the knee joint with the total volume of 20 μl of solution containing 1, 10 or 100 μg of cytochrome *c* in the first experiment and 10 μg in the following experiments. Control mice received an equivalent volume of control buffer.

In vivo cell depletion procedures

Monocyte depletion. Etoposide was purchased from Bristol Myers Squibb (Bromma, Sweden) and diluted 1 : 10 in PBS (0.13 M NaCl, 10 mM sodium phosphate, pH 7.4) from a stock solution of 20 mg/ml. Etoposide is a drug which has been shown to deplete selectively the monocyte population in mice [16, 17]. BALB/c mice were injected subcutaneously with 70–150 μl of etoposide (corresponding to 12.5 mg/kg body weight [17]) once a day during the experiment, starting 2 days before the i.a. injection of cytochrome *c*. Mice were monitored and weighted individually once a day and the dose of etoposide was adjusted to body weight. Results of the flow cytometric (FACScan flow cytometer, Becton Dickinson, San Jose, CA) analysis demonstrated that monocytes decreased more 80% compared with the levels in untreated animals; this has been demonstrated previously [15, 17].

Neutrophil depletion. The hybridoma cells secreting monoclonal antibody RB6-8C5 were a kind gift from Dr R. Coffmann (DNAX Research Institute, Palo Alto, CA, USA). The antibody

was produced and purified as described in detail previously [18]. Monoclonal antibody (mAb) RB6-8C5 is a rat immunoglobulin (IgG2b) that selectively depletes mature mouse neutrophils. BALB/c mice were injected intraperitoneally with 1 mg of RB6-8C5 mAb 16 h before the i.a. administration of cytochrome *c*. Anti-neutrophil mAb RB6-8C5 depletes the granulocyte population by up to 90% [15, 18].

Haematological analyses

To assess the effect of cell depletion, the mice were bled from tail vein into heparinized tubes and total leucocyte counts were determined in a cell counter (Sysmex KX-21; Toa Medical Electronics, Kobe, Japan). To evaluate the percentages of lymphocytes, monocytes and granulocytes, peripheral blood leucocytes (10^4 cells per sample) were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The absolute numbers of different leucocyte subsets were then calculated from the total leucocyte counts.

Histopathological examination

Three and 10 days after the i.a. injection of cytochrome *c* or control buffer, the mice were killed and knee joints were removed, fixed in 4% paraformaldehyde, decalcified and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin. All the slides were coded and assessed in a blinded manner by three observers with regard to synovial hypertrophy (membrane thickness more than two layers), pannus formation (synovial tissue overlaying joint cartilage) and cartilage and bone destruction [19]. The extent of synovitis was judged on a scale from 0 to 3 (0 = no signs of inflammation; 1 = mild synovial hypertrophy with up to five cell layers; 2 = moderate inflammation characterized by hyperplasia of synovial membrane up to 10 cell layers and influx of inflammatory cells throughout the synovial tissue; 3 = marked synovial hypertrophy with more than 10 cell layers, the synovial tissue infiltrated by inflammatory cells).

Immunohistochemical evaluation

Three days after the i.a. injection of cytochrome *c* and control buffer, the mice were killed and knee joints were removed and demineralized by a procedure detailed in an earlier report [20]. The demineralized specimens were then embedded (Tissue-Tek; Miles, Elkhart, IN, USA), frozen in isopentane prechilled with liquid nitrogen and kept at −80°C until cryosectioned. Sections (5 μm) were cut sagittally, fixed in cold acetone for 5 min, washed in PBS and then incubated for 20 min at 37°C in glucose oxidase buffer solution to deplete endogenous peroxidase. Unlabelled primary rat monoclonal anti-CD11b (Mac-1, clone M1/70), anti-CD4 (H129.19, [21]), anti-CD8 (Lyt 2, BD Biosciences Pharmingen, San Diego, CA, USA) were used as primary antibodies and rat IgG as a negative control at appropriate dilutions. Biotin-labelled rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody, and was followed by incubation with StreptABComplexes (Dako, Glostrup, Denmark) and 3-amino-9-ethyl carbazole buffer containing H_2O_2. All sections were counterstained with Mayer's haematoxylin.

Nuclear extracts

Spleens were obtained from healthy NMRI mice and splenocyte cultures were prepared as described previously [17]. The cells (2×10^6/ml) were incubated for 2 h at 37°C, 5 ml/well in a six-well microtitre plate with different concentrations of cytochrome *c* or 10 μg/ml of LPS as a positive control. Stimulation was stopped

with ice-chilled PBS; the cell suspension was washed twice with ice-chilled PBS, resuspended in 2 ml of hypoton buffer (10 mM HEPES, pH 7.9, at 4°C, 0.1 mM ethylenediamine tetraacetate (EDTA), 0.1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetra acetic acid (EGTA), 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermin, 1 M dithiothreitol (DTT) + proteinase inhibitors [Complete^{TM} Mini Tablets, 1 tablet/10 ml]) and homogenized. Following centrifugation at 4°C 14 000 *g* for 10 min, the supernatant was carefully removed and the pellet was resuspended in ice-cold extraction buffer (20 mM HEPES, pH 7.9, at 4°C, 25% glycerol, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 M DTT + proteinase inhibitors [Complete^{TM} Mini Tablets, 1 tablet/10 ml]). After extraction at 4°C overnight on a rotator, the tubes were centrifuged at 4°C 14 000 *g* for 1 h. The supernatant was collected and stored at −80°C in aliquots until the analysis of NF-κB could be performed. Before freezing, the protein concentration was determined using the Bradford reagent (Sigma).

Electrophoretic mobility shift assay (EMSA)

The EMSA was performed as described elsewhere [22] with minor modifications. The sequences of synthetic oligonucleotides used for the NF-κB probes were as follows: NF-κB sense, 5′-GGC TCA AAC AGG GGG CTT TCC CTC CTC CAAT AT-3′; NF-κB anti-sense, 5′-GGA TAT TGA GGA GGG AAA GCC CCC TGT TT G AG-3′). Oligonucleotides were annealed at 56°C. The double-stranded product was purified by eluation from the electrophoretic gel. Double-stranded oligonucleotides were labelled with α[^{32}P]-labelled deoxynucleotide (Amersham Pharmacia Biotech, Uppsala, Sweden) using Klenow polymerase (Roche Diagnostics, Mannheim, Germany) and used in the binding reaction with the nuclear protein extracts.

Binding reaction mixtures in a volume of 20 μl, containing an equal amount of nuclear protein, 5 μg of poly(dI-dC)poly(dI-dC) (Amersham Pharmacia Biotech), 1 μl [^{32}P]-labelled probe containing NF-κB binding sites, 1 mM DTT, 0.2 mg/ml bovine serum albumin and binding buffer (0.02 M Tris–HCl, pH 7.9, 30 mM NaCl, 5 μM EGTA, 5% glycerol) were mixed and incubated for 20 min at room temperature. The final NaCl concentration was adjusted to 80 mM in all samples. The complexes were separated by electrophoresis through a native 5% polyacrylamide gel containing 0.25 × TRIS–borate–EDTA buffer, 3% glycerol and 0.1% ammonium persulphate. The gel was vacuum-dried and exposed to X-ray film for 48 h at −80°C. For the competition assay, the nuclear extract was preincubated with an excess of unlabelled NF-κB oligonucleotide for 10 min on ice prior to the addition of the radiolabelled probe. For supershift assay, nuclear extract was incubated with 1 μl of p50 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 20 min on ice prior to EMSA.

In vitro stimulation of spleen cells

Spleens from healthy BALB/c mice were aseptically removed, passed through a nylon mesh and washed with PBS. Erythrocytes were depleted by lysis with ammonium chloride. The cells (2 × 10^6/ml) were then cultured for 48 h at 37°C in Iscove's complete medium [5 × 10^{-5} M mercaptoethanol, 10% heat-inactivated fetal calf serum (Integro, Leuvenheim, The Netherlands), 1% L-glutamine and 50 μg/ml of gentamicin] with cytochrome *c* at final concentrations of 0, 0.01, 0.1, 1, 10 and 100 μg/ml, and cell culture supernatants were collected for determination of cytokine and chemokine levels. Experiments were run in duplicate and the values are reported as the mean ± s.e.m. for four mice.

For functional inhibition of NF-κB, spleen cells from four healthy male NMRI mice were prepared as described above. The

cells (2 × 10^6/ml) were cultured for 3 h at 37°C with the specific NF-κB inhibitor parthenolide [23] at concentration 1 μg/ml and then stimulated with 100 μg/ml of cytochrome *c*. Cell culture supernatants were collected after 24 h and IL-6 levels were measured by ELISA (Quantikine$^®$ mouse kit; R&D Systems, Minneapolis, MN, USA). Using the trypan blue dye exclusion test, we observed that the viability of the cells after 24 h remained > 70% in all treatment groups (cells treated either with parthenolide alone or in combination with cytochrome *c* or unstimulated).

Determination of cytokine and chemokine levels

Macrophage inflammatory protein 2 (MIP-2), MIP-1α, TNF-α, monocyte chemoattractant protein 1 (MCP-1) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) levels were measured using specific Quantikine mouse kits (R&D Systems) according to the manufacturer's protocol.

For the measurement of IL-6, subclone B9 of the murine hybridoma cell line B13.29 was employed. This cell line is dependent on IL-6 for its growth [24, 25]. B9 cells were harvested from tissue culture flasks, washed in IL-6-free medium, seeded into microtitre plates (Nunc, Roskilde, Denmark) at 5000 cells/ well, and cultured in Iscove's complete medium (supplemented with 5 × 10^{-5} M mercaptoethanol, 10% fetal calf serum, 1% L-glutamine and 50 μg/ml of gentamicin) together with samples. After 68 h, 1 μCi of [^{3}H] thymidine (Amersham) was added and the cells were harvested 4 h later onto filters and counted in a beta counter. The triplicate samples were tested at different dilutions and compared with a recombinant IL-6 standard (Genzyme, Cambridge, MA, USA). The IL-6 level in culture supernatants from cells treated with parthenolide was determined using an ELISA kit.

Statistical analysis

Differences between groups with respect to arthritis severity and arthritis frequency were calculated using the Kruskal–Wallis test followed by the Mann–Whitney test or Fisher's exact probability test respectively. Correlations between different variables in patients were assessed with the Spearman rank correlation test. All values are reported as the mean ± s.e.m. or s.d. *P* values below 0.05 were considered significant.

The clinical investigation was approved by the ethical committee of Göteborg University and informed consent was obtained from all patients.

Results

Cytochrome c induces arthritis in the healthy host

To evaluate the potential inflammatogenic role of cytochrome *c*, NMRI mice were given a single i.a. injection of this protein. The cytochrome *c* dose chosen was determined in a pilot experiment in which 0, 1, 10 and 100 μg of the protein were injected i.a. into mouse knee joints. Histopathologically verifiable arthritis was detected in 75% of mice given 10 μg but only in 25% of mice given 1 or 100 μg of cytochrome *c* (data not shown). The optimal dose of 10 μg was chosen for further experiments. To assess the duration of arthritis caused by cytochrome *c*, mice were injected i.a. and followed for 3 and 10 days. Mild arthritis appeared in 75% of mice 3 days after injection but its frequency decreased to 20% after another 8 days (Fig. 1). Controls injected with buffer showed 20 and 0% of arthritis respectively (Fig. 1A). To ascertain the specificity of arthritis triggered by cytochrome *c*, 10 μg of mouse serum albumin as irrelevant protein was injected into the knee joint of NMRI mice. Only minor inflammatory infiltrates were seen in 20% of mice, comparable with the percentage obtained with
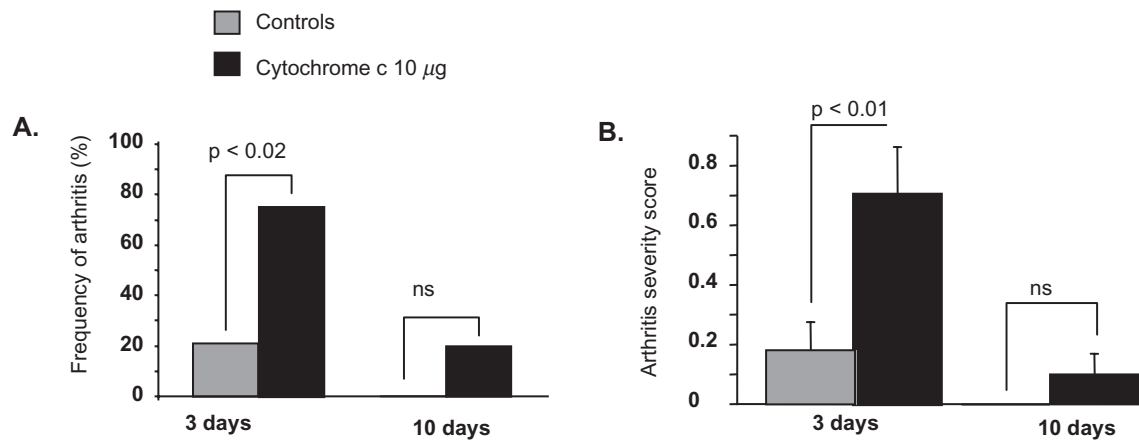
FIG. 1. (A) Incidence (%) and (B) severity (mean $\pm$ S.E.M.) of arthritis in 43 NMRI mice following i.a. administration of either $10\,\mu\mathrm{g}$ of cytochrome *c* (22 animals) or control buffer (21 animals). The synovitis was judged on a scale from 0 to 3 in a blinded manner by three observers (see Materials and methods). The statistical significance of the frequency of arthritis calculated using Fisher's exact probability test and differences between groups with respect to arthritis severity were tested for significance using the Mann–Whitney test.
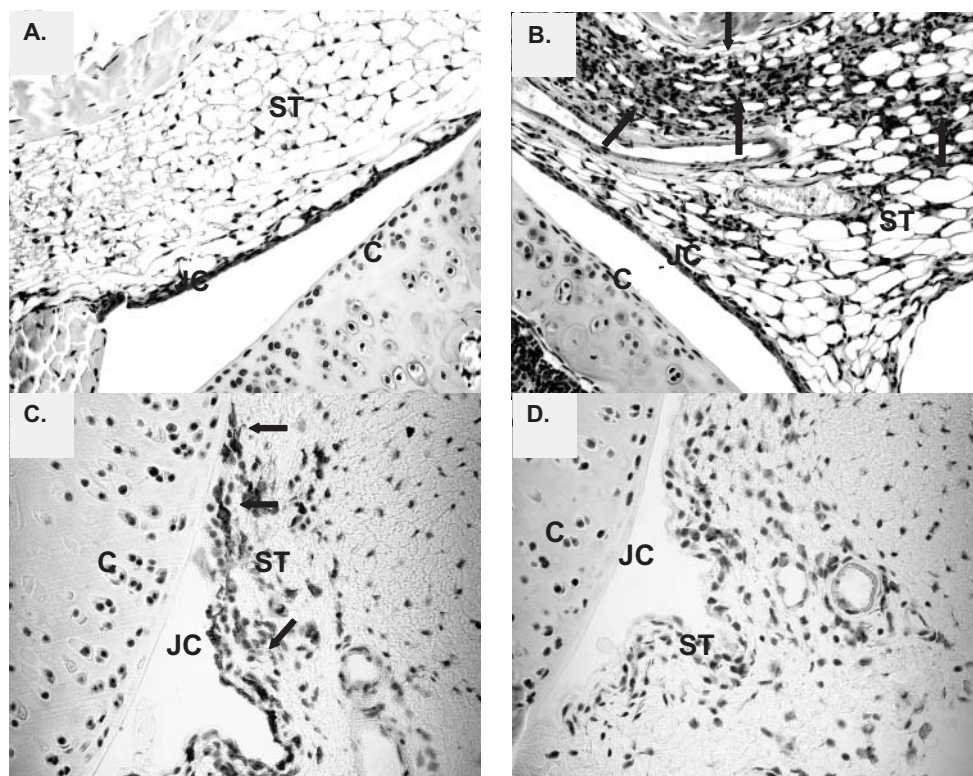

FIG. 2. (A, B) Histology of mouse knee joints obtained 3 days after i.a. injection of (A) control buffer or (B) $10\,\mu\mathrm{g}$ of cytochrome *c*. Arrows indicate the presence of inflammatory cells into synovial tissue. Original magnification $\times 200$. (C, D) Immunohistochemical staining (C) with Mac-1 antibody and (D) control rat IgG of mouse joint obtained on day 3 after i.a. injection of $10\,\mu\mathrm{g}$ of cytochrome *c*. Arrows point to positively stained Mac-1 cells. Original magnification $\times 400$. C, cartilage; JC, joint cavity; ST, synovial tissue. This figure may be viewed in colour as supplementary data at *Rheumatology* Online.

buffer. This indicates that cytochrome *c* elicits an acute but short-lasting inflammatory response.

The synovitis (Fig. 2B) was characterized by influx of inflammatory cells throughout the synovial tissue. Neither cartilage erosions nor pannus formation was observed.

Immunohistochemical analysis of joints obtained on day 3 after i.a. injection of $10\,\mu\mathrm{g}$ of cytochrome *c* revealed that most inflammatory cells in the synovium stained with Mac-1, which is a marker of granulocytes and macrophages (Fig. 2C, D). In contrast, CD4^{+} and CD8^{+} lymphocytes were not detected in the

inflammatory infiltrates. The above data suggest that the innate immune system has a major role in the inflammatory process caused by cytochrome *c*.

Neutrophils are key mediators in cytochrome c-induced arthritis

To further ascertain the role of Mac-1-positive immune cells in arthritis induced by cytochrome *c*, we carried out cell depletion
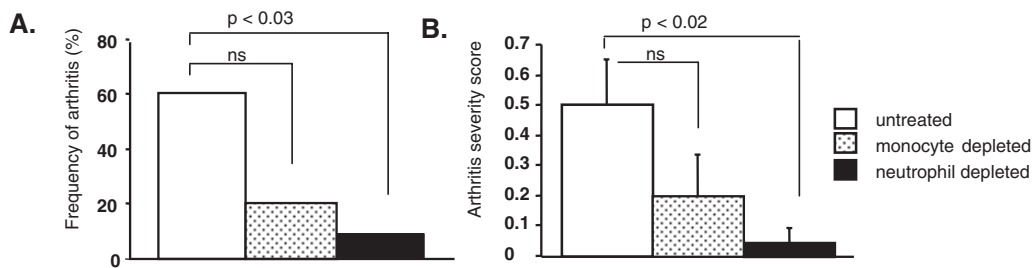
FIG. 3. Effects of neutrophil and monocyte depletion on the (A) incidence and (B) severity of arthritis in BALB/c mice after i.a. injection of $10\,\mu g$ of cytochrome *c*. For monocyte depletion, mice were injected subcutaneously with $70–150\,\mu l$ of etoposide (corresponding to $12.5\,mg/kg$ body weight) once a day during the experiment, starting 2 days before the i.a. injection of cytochrome *c*. Mice were monitored and weighed individually and the dose of etoposide was adjusted to body weight. For neutrophil depletion, 1 mg of neutrophil-specific RB6-8C5 mAb was injected before the i.a. administration of cytochrome *c*. Each bar represents the average of 10 or 11 animals. The statistical significance of arthritis frequency was calculated using Fisher's exact probability test and differences between groups with respect to arthritis severity were tested for significance using the Mann–Whitney test. Values are reported as mean $\pm$ s.e.m. for arthritis severity score and as percentages for incidence.

procedures *in vivo*. BALB/c mice were pretreated with either etoposide or RB6-8C5 mAb, leading to the selective depletion of the monocyte or neutrophil population respectively. Interestingly, depletion of neutrophils significantly abrogated the incidence as well as the severity of arthritis (Fig. 3) compared with untreated littermates. Only one mouse out of 11 had a minor inflammatory infiltrate in the synovial tissue in the neutrophil-depleted group, whereas 60% of the untreated controls displayed signs of inflammation (Fig. 3A). The reduction in the severity and frequency of arthritis in monocyte-depleted mice was less pronounced.

Cytochrome c activates transcription factor NF-κB

Transcription factor NF-κB is known to control and regulate the gene expression of many proinflammatory cytokines and chemokines at the transcriptional level [26, 27]. We determined whether the exposure of mouse spleen cells to different concentrations of cytochrome *c* (0, 1, 10 and $100\,\mu g/ml$) led to the activation of NF-κB. As detected by the electrophoretic mobility shift assay (Fig. 4), stimulation of cells with $10\,\mu g/ml$ of cytochrome *c* resulted in a remarkable activation of transcription factor NF-κB, which was similar to that induced by $10\,\mu g/ml$ of LPS. Incubation of cells with $1\,\mu g/ml$ or $100\,\mu g/ml$ of cytochrome *c* also activated NF-κB, but to a lesser degree. These data show clear parallelism with the clinical data regarding the dose required for the induction of cytochrome *c* arthritis.

Cytochrome c triggers the release of proinflammatory cytokines and chemokines in vitro

Since cytokines have been shown to play an important role in the pathogenesis of arthritis, we decided to analyse the cellular effects of exposure to cytochrome *c in vitro*. Mouse splenocytes were stimulated for 48 h with different concentrations of cytochrome *c* ranging from 0.01 to $100\,\mu g/ml$, and neutrophil/macrophage-associated cytokines and chemokines were analysed in cell culture supernatants. Cytochrome *c* dose-dependently induced the release of activators of monocytes (MCP-1, MIP-1α, RANTES), neutrophils (MIP-2), as well as the production of proinflammatory cytokines, such as TNF-α and IL-6 (Fig. 5).

The relationship of cytokine expression and NF-κB activation induced by cytochrome *c* was further investigated employing a selective NF-κB inhibitor parthenolide [23]. Mouse splenocytes were pretreated for 3 h with $1\,\mu g/ml$ of parthenolide before stimulation with $100\,\mu g/ml$ of cytochrome *c*, and IL-6 levels were
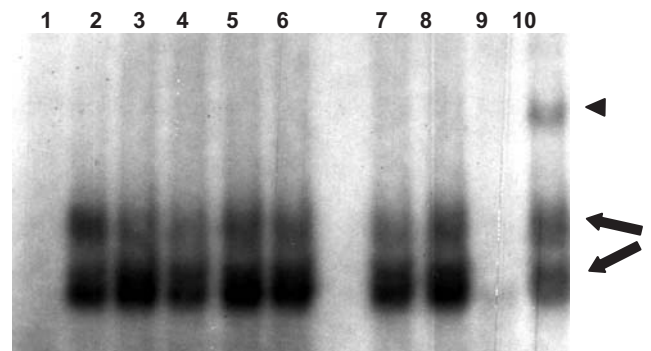

FIG. 4. Cytochrome *c* activates NF-κB in mouse spleen cells. An image of an EMSA gel showing the NF-κB activation in mouse spleen cells after 2 h of incubation with $0\,\mu g/ml$ (lanes 3 and 7), $1\,\mu g/ml$ (lane 4), $10\,\mu g/ml$ (lanes 5 and 8) and $100\,\mu g/ml$ (lane 6) of cytochrome *c*. Nuclear extract obtained from spleen cells stimulated with $10\,\mu g/ml$ of LPS is shown as a positive control (lane 2). Free probe is also shown (lane 1). For supershift and cold competition experiment (lanes 7–10), nuclear extracts from spleen cells were left unstimulated (lane 7) or stimulated for 2 h with $10\,\mu g/ml$ of cytochrome *c* (lanes 8–10) and incubated in the absence (lane 8) or presence of an excess of homologous oligonucleotide (lane 9), or subjected to supershift with antibody specific to p50 subunit (lane 10). The arrowhead indicates a supershifted band and arrows show NF-κB complexes. Spleen cells were obtained from three mice and the experiment was repeated with similar results.

measured in culture supernatants after 24 h. Our results show that preincubation of the cells with parthenolide inhibited cytochrome *c*-triggered IL-6 release by an average of $85 \pm 11\%$ (s.d.) compared with cytochrome *c* treatment alone.

Cytochrome c levels in synovial fluid and sera of patients with RA

In the first series of experiments, we addressed the question of whether cytochrome *c* can be detected in the synovial fluid and blood of RA patients. We used sera and synovial fluid from 32 RA patients. Twenty-two patients had erosive arthritis and 28 were RF-positive. Sera from 16 healthy individuals (mean age 50 ± 7 yr) were also assessed with respect to cytochrome *c* levels. The level of cytochrome *c* in sera from RA patients ($1.10 \pm 0.25\,ng/ml$) was significantly lower ($P < 0.002$) compared with sera of healthy
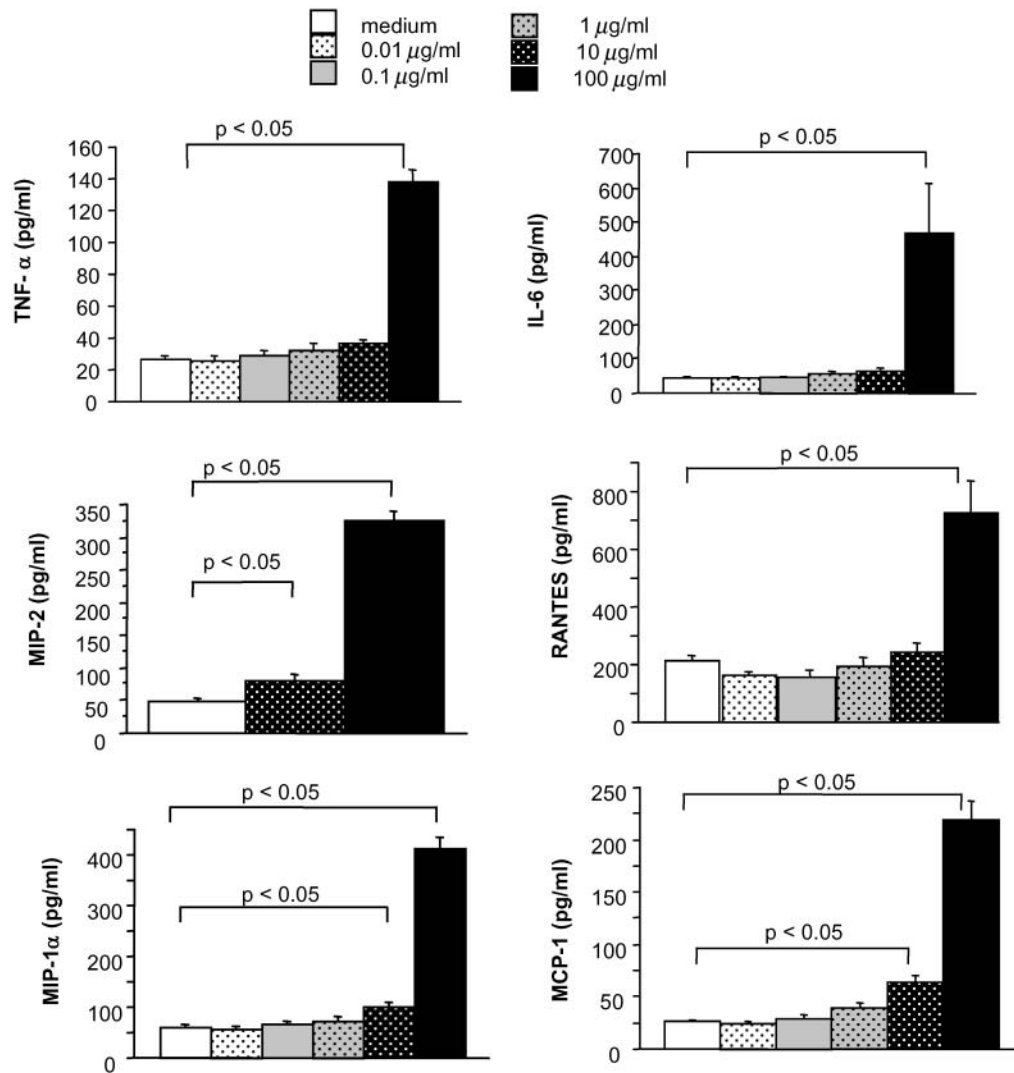
FIG. 5. *In vitro* induction of cytokines and chemokines by extracellular cytochrome *c*. Unfractionated mouse spleen cells were incubated with increasing amounts of cytochrome *c* for 48 h. Culture supernatants were analysed by ELISA (TNF-α, MIP-2, RANTES, MCP-1, MIP-1α) or bioassay (IL-6). Experiments were run in duplicate or triplicate (IL-6) and the values are reported as mean ± s.e.m. of four mice.

controls (1.88 ± 0.26 ng/ml). Further, cytochrome *c* levels in synovial fluid of RA patients were half as low (0.49 ± 0.07 ng/ml) as in corresponding blood samples ($P < 0.02$) (Fig. 6). No differences with respect to cytochrome *c* were found in patients having erosive *vs* non-erosive disease. The concentration of cytochrome *c* in sera of arthritis patients decreased with ageing, showing a weak correlation ($r = -0.37$, $P < 0.05$), which was not observed in the blood of healthy subjects. No correlation was found between cytochrome *c* levels in synovial fluid with respect to disease duration, the age of patients, or the levels of C-reactive protein.

Discussion

This is the first report providing evidence that cytochrome *c* triggers synovitis in a healthy host and may therefore play a role in the pathogenesis of arthritis. Indeed, a single i.a. injection of cytochrome *c* into a mouse knee joint induced an acute inflammatory synovitis. In this setting, the innate immune system plays a major role. This conclusion is based on the findings that T cells could not be found in the inflamed synovium. In contrast, most cells were Mac-1 positive, i.e. they had the phenotype of neutrophils and macrophages. Further, depletion of neutrophils and, to some extent, monocytes significantly abrogated the incidence and severity of inflammation, indicating the involvement of these cell types in the inflammatory process. The synovitis triggered by cytochrome *c* was rather short-lasting—by day 10 the incidence of arthritis had decreased to 55% again, indicating lack of acquired immunity as a driving force.

Cytokines and chemokines have been shown to play an important role in the pathogenesis of arthritis. The ability of cytochrome *c* to induce the release of proinflammatory cytokines and chemokines from cells surely contributes to the pathogenesis of the inflammatory process inside the joint. In our study, we have shown for the first time that *in vitro* stimulation of splenocytes with cytochrome *c* leads to a remarkable release of inflammatory mediators. These proinflammatory molecules have also been found in the synovial tissue of arthritic patients and have been shown to regulate the inflammatory process in the joints [28]. The chemokines measured, RANTES, MIP-1α and MCP-1, are known as the strongest chemoattractants for monocytes [29–32], whereas MIP-2 exerts neutrophil chemotactic activity.
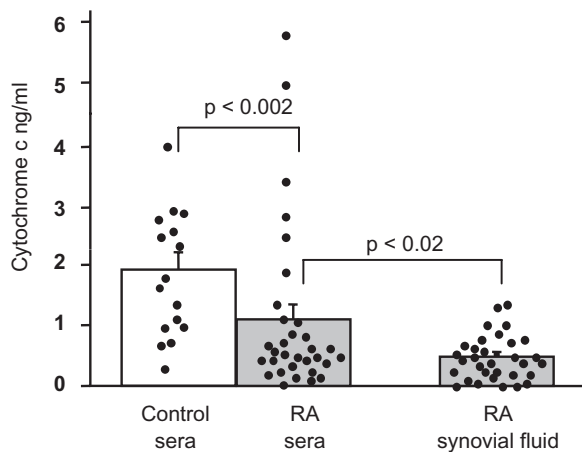
FIG. 6. Levels of cytochrome *c* in sera and synovial fluid of RA patients and sera of healthy controls. Synovial fluid and blood samples were collected from 32 patients who met the American College of Rheumatology criteria for RA (mean disease duration 14 ± 9 yr). Blood samples from 16 healthy individuals were used as a control. Statistical differences with respect to cytochrome *c* levels in sera were calculated using the Mann–Whitney test. The paired sample *t*-test was used to calculate differences in cytochrome *c* levels in RA patients.

NF-κB, a transcription factor that translocates from the cytoplasm into the nucleus in response to various stimuli, activates the transcription of many genes [33]. In our study, we show that stimulation of cells with $10\,\mu$g/ml of cytochrome *c* resulted in the activation of NF-κB, a prerequisite for the production of many proinflammatory cytokines involved in the induction of arthritis. This suggests that the proinflammatory effect of cytochrome *c* might be, at least in part, mediated through NF-κB activation. Interestingly, an even higher concentration of cytochrome *c* ($100\,\mu$g/ml) had a less pronounced effect on NF-κB activation. These results are in line with our *in vivo* experiments showing bimodal effects of cytochrome *c* on the induction of arthritis. Furthermore, we tested the relationship of cytokine release and NF-κB activation using a specific NF-κB inhibitor (parthenolide) and found that inhibition of NF-κB led to an 85% decrease in cytochrome *c*-triggered IL-6 release. This further supports the involvement of the NF-κB pathway. Recent data suggest that NF-κB subunits play a distinct role in the pathogenesis of inflammatory arthritis [34], and it is likely that the different forms of NF-κB activate different sets of target genes. It has been shown that p50-deficient mice are refractory to the induction of both chronic and acute arthritis, showing that this subunit is essential for local joint inflammation and destruction [34]. In our study, we confirmed the activation of p50 subunit by EMSA supershift as a consequence of exposure of inflammatory cells to cytochrome *c*.

The concentrations used in our *in vitro* study can be easily found in the blood of patients during certain diseases. Indeed, the levels of cytochrome *c* in the serum of patients following myocardial infarction were in the range of 10–250 μg/ml [11]. Further, increased cytochrome *c* levels were observed in serum from patients with haematological malignancies; they were up to 2- to 8-fold higher than in healthy controls [10]. In contrast, our results indicate decreased concentrations of cytochrome *c* in the sera of RA patients compared with those in healthy controls. Importantly, the cytochrome *c* levels found in the synovial fluid of RA patients were strikingly low when compared with matching blood samples. How would low i.a. levels of cytochrome *c* be viewed from the perspective of its *in vivo* proinflammatory properties? A known precedent in this case is the complement system. Indeed, activation of the complement system is a well-established part of the inflammatory cascade in arthritis [35]. However, levels of

complement factors in the synovial fluid of RA patients are significantly lower than in the circulation [36]. In the cases of both complement components and cytochrome *c*, these findings are considered to be due to consumption during the inflammatory process. In the case of cytochrome *c*, low i.a. levels of this molecule could reflect its function as an antioxidant in mediating superoxide removal [37, 38]. In addition, considering cytochrome *c* as marker of cell death, our results indicate the decreased apoptosis locally in the inflamed joints of patients with RA and are in line with recent findings showing the important role of impaired apoptosis in the pathogenesis of RA [14].

In conclusion, the physiological relevance of extracellular cytochrome *c* is not clear. Our results indicate that it may act as a trigger of inflammation leading to NF-κB activation and may be followed by the release of cytokines and chemokines. Released active mediators recruit monocytes and neutrophils, and activation of the anti-apoptotic pathway mediated by NF-κB limits cell death to prevent further tissue damage. In addition, considering cytochrome *c* as a marker of cell death, our results indicate that apoptosis is decreased locally in the inflamed joints of patients with RA.

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