Effects of RWJ 67657, a p38 mitogen activated protein kinase (MAPK) inhibitor, on the production of inflammatory mediators by rheumatoid synovial fibroblasts

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Objective: To investigate the effect of the p38 mitogen activated protein kinase (MAPK) inhibitor RWJ 67657 on inflammatory mediator production by rheumatoid synovial fibroblasts (RSF).

Methods: RSF were pretreated with RWJ 67657 and stimulated with TNFα and/or IL-1β. Protein levels and mRNA expression of MMP-1, MMP-3, TIMP-1, IL-6, and IL-8 were determined, as was mRNA expression of COX-2 and ADAMTS-4.

Results: MMP-3 production was significantly inhibited at 1 μM RWJ 67657 and MMP-1 production at 10 μM, while TIMP-1 production was not inhibited. Inhibition of IL-6 and IL-8 protein production was seen at 0.1 μM RWJ 67657. Expression profiles of mRNA were in accordance with protein production. Inhibition of COX-2 mRNA expression occurred at 0.01 μM RWJ 67657.

Conclusions: RWJ 67657 inhibits major proinflammatory mediator production in stimulated RSF at pharmacologically relevant concentrations. These findings could have important relevance for the treatment of rheumatoid arthritis.

The pathogenesis of rheumatoid arthritis involves complex interrelations between T cells, macrophages, fibroblasts, and other immune cells. Growing evidence suggests that activated rheumatoid synovial fibroblasts (RSF) play a major role in both initiating and driving rheumatoid arthritis.1 In particular, RSF in the lining layer display numerous features of cellular activation which ultimately result in aggressive invasive behaviour. These cells can attach to the articular cartilage and invade the extracellular matrix. Furthermore, RSF are important producers of inflammatory mediators such as cytokines and matrix-metalloproteinases (MMP). Many of these mediators are regulated by mitogen activated protein kinase (MAPK) pathways and downstream transcription factors.2 At least three subgroups of MAPK have been identified. These are the extracellular signal regulated kinases (ERK), the c-Jun N-terminal or stress activated protein kinases (JNK/SAPK), and the p38 MAPK.3 In general ERK are activated by growth factors and hormones, whereas both JNK and p38 MAPK are activated by environmental stress and inflammatory cytokines.4

The involvement of p38 MAPK in the production of inflammatory mediators by fibroblasts has been reported in recent years. The role of p38 MAPK in relation to interleukin-6 (IL-6) and interleukin-8 (IL-8) production has been established in RSF.5 Also, involvement of p38 MAPK in MMP production has been demonstrated in dermal6 and gingival fibroblasts.7 Another matrix degrading enzyme, aggrecanase-1 or ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motif), induced by cytokines in RSF, is important in cartilage degradation in rheumatoid arthritis.8 However, its signal transduction pathways are not known at the moment.

Prostaglandins have also been described as being under the influence of p38 MAPK.9 This has been confirmed in a study in which it was reported that glucocorticoids destabilise cyclo-oxygenase-2 (COX-2) mRNA by inhibiting the p38 MAPK route.10

Interest in protein kinases as drug targets has increased in the recent years; in particular, p38 MAPK inhibitors have been developed11 because p38 plays an important role as a major signal transducer responding to cellular stress stimuli such as cytokines. Because the production of interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) is influenced by p38 MAPK, p38 MAPK inhibitors are expected to inhibit not only the production of these principal proinflammatory cytokines but also their subsequent actions, leading to interruption of the vicious cycle that often occurs in inflammatory diseases. The use of p38 MAPK inhibitors could therefore provide an important advantage in treatment.

The p38 MAPK inhibitor RWJ 67657 (4-[[4-(4-fluorophenyl)-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-3-butyryl-1-ol) has been shown to inhibit the release of TNFα from lipopolysaccharide treated human peripheral blood mononuclear cells, with an IC50 of 3 nM, as well as inhibiting the release of TNFα from peripheral blood mononuclear cells treated with the super antigen staphylococcal enterotoxin B.14 This compound was approximately 10-fold more potent than the reference standard p38 MAPK inhibitor SB 203580 in all p38 dependent in vitro systems tested. RWJ 67657 specifically inhibited the enzymatic activity of recombinant p38 α and β, but not γ and δ, in vitro, and had no significant activity against a variety of other kinases.14 Furthermore it was reported that this compound suppressed clinical and cytokine responses to endotoxin in healthy human volunteers.13 Recently a study was published on the pharmacokinetics and pharmacodynamics of RWJ 67657 in humans.16 This study showed acceptable safety and pharmacokinetic characteristics, warranting further investigation in a repeat-dose setting.

In the present study we investigated the effects of RWJ 67657 on the release of proinflammatory mediators produced by activated RSF.20

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin-1 motif; COX-2, cyclo-oxygenase-2; JNK, c-Jun N-terminal protein kinase; ERK, extracellular signal regulated kinase; IL, interleukin; MAPK, mitogen activated protein kinase; MMP, matrix-metalloproteinase; RSF, rheumatoid synovial fibroblast; SAPK, stress activated protein kinases; TIMP-1, tissue inhibitor of matrix-metalloproteinases; TNF, tumour necrosis factor

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Accepted 24 January 2004

by RSF, after stimulation with IL-1β or TNFα or both. Collagenase-1 (MMP-1), stromelysin-1 (MMP-3), and the tissue inhibitor of matrix-metalloproteinases (TIMP-1) were studied at both protein and mRNA expression level. The same was done for IL-6 and IL-8. In addition we looked at the effects on mRNA expression levels of aggrecanase-1 (ADAMTS-4) and COX-2.

METHODS
Reagents
RWJ 67657 was provided by Johnson and Johnson (RW Johnson Pharmaceutical Research Institute, Raritan, New Jersey, USA). Recombinant human IL-1β and recombinant human TNFα were purchased from R&D Systems (Minneapolis, Minnesota, USA). Fetal calf serum (FCS) and Dulbecco’s modified Eagle medium (DMEM) were obtained from Biowhittaker (Verviers, Belgium). Anti-CD14 antibodies were from IQP (Groningen, Netherlands) and anti-fibroblast antibodies (clone 5B5) from Dako (Glostrup, Denmark). All reagents for RNA isolation and reverse transcriptase reaction were obtained from Invitrogen, Life Technologies (Gaithersburg, Maryland, USA). Reagents for real time reverse transcriptase polymerase chain reaction (RT-PCR) were obtained from Applied Biosystems (Foster City, California, USA). Specific antibodies to p38 MAPK, phospho-p38 MAPK, and phospho-MAPKAPK-2 were purchased from Cell Signalling Technologies (Beverly, Massachusetts, USA).

Isolation and culture of rheumatoid synovial fibroblasts
Synovial fibroblasts were isolated from synovium from eight patients with rheumatoid arthritis who underwent total joint replacement. Synovium was minced and digested with...
1 mg/ml collagenase (type 1A, Sigma, Zwijndrecht, Netherlands) in DMEM (with L-glutamine and gentamicin) for two hours at 37°C. The cell suspension was filtered through a cell strainer (70 µm) (Beckton Dickinson, Franklin Lakes, New Jersey, USA) and washed with phosphate buffered saline. Cells were cultured in a 5% CO₂/37°C incubator in DMEM with 10% FCS, and non-adherent cells were discarded after overnight incubation. At passage 3, the cell population consisted of CD14 negative/5B5 positive cells (fibroblast-like synoviocytes) and these cells were used for experiments until passage 8.

For all experiments the cells were plated in 6-well or 48-well plates and serum starved for 24 hours in DMEM+1% FCS to synchronise cells in a non-activating and non-proliferating phase. Next they were pretreated with increasing concentrations (0.001 µM to 10 µM) of RWJ 67657 (stock solution 10 mM in DMSO) for one hour before stimulation with 1 ng/ml IL-1β and/or TNFα.

Determinant of MMP-1, MMP-3, TIMP-1, IL-6, and IL-8 levels in cell culture supernatants

Confluent synovial fibroblasts (n = 5) were plated in 48-well plates (10 000 cells/ml per well) and treated as above. After 48 hours’ stimulation, supernatants were harvested and concentrations of MMP-1, MMP-3, TIMP-1, IL-6, and IL-8 were determined using enzyme linked immunosorbent assays (ELISAs) developed in our laboratory. The MMP-3 ELISA has been described previously. The MMP-1 ELISA was used monoclonal anti-MMP-1 (clone 63665.111) and biotinylated goat anti-human MMP-1 (both from R&D Systems). The TIMP-1 antibodies (R&D Systems) in the ELISA were monoclonal anti-TIMP-1 (clone 63515.111) and biotinylated goat anti-human TIMP-1. The detection of the biotinylated antibodies was done with streptavidin-poly-HRP (CLB, Netherlands) and TMB colour reaction.

RNA isolation

Synovial fibroblasts (n = 6) were plated in 6-well plates (0.5 x 10⁶ cells/well/4 ml) and treated as above. After six or 24 hours of stimulation, total RNA was isolated from the cells with Trizol reagent according to the manufacturer’s instructions (Life Technologies). After DNase treatment (DNA-free, Ambion, Austin, Texas, USA) cDNA was synthesised from 2.0 µg of total RNA using M-MLV reverse transcriptase and oligo (dt)₁₅.

Real time RT-PCR

For quantitative detection of mRNA expression a fluorescence based real time RT-PCR was undertaken, which allows relative quantification of steady state mRNA. The amount of emitted fluorescence is proportional to the amount of PCR product and enables monitoring of the PCR reaction. For the measurement of MMP-1, MMP-3, TIMP-1, ADAMTS-4, IL-6, IL-8, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1 µl of cDNA in duplicate was used for amplification by the real time quantitative PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with specific Taqman primers/probes.

The amount of target, normalised to an endogenous reference and relative to a calibrator, is given by:

\[ \text{DD}_{	ext{CT}} = \text{CT}_{	ext{target}} - \text{CT}_{	ext{endogenous}} \]

where DD is the difference in threshold cycle (CT), \( \text{DD}_{\text{CT}} \) is the threshold cycle, \( \text{DD}_{\text{CT}} \) is the threshold cycle of the target, \( \text{DD}_{\text{CT}} \) is the threshold cycle of the endogenous reference, and \( \text{DD}_{\text{CT}} \) is the threshold cycle of the calibrator.

The results are expressed as fold induction relative to untreated samples.

Western blotting to detect phosphorylation of p38 MAPK and MAPKAPK-2

Phosphorylation of p38 MAPK was analysed by western blotting. Synovial fibroblasts were plated in 6-well plates (0.5 x 10⁶ cells/well/4 ml) and treated as above. After stimulation with TNFα and/or IL-1β for up to 60 minutes, cell extracts were prepared by lysing the cells with 1x SDS sample buffer (containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris-HCl (pH = 6.8), and 0.01% bromophenol blue). Cells were scraped off the wells and the lysates were subsequently sonicated for 5–10 seconds and boiled for five minutes. After centrifugation the samples were loaded onto a 10% SDS-PAGE gel and resolved by running at 200 V and 15 W constant. Semidry blotting onto a nitrocellulose membrane was followed by immunoblotting with specific antibodies to p38 MAPK, phospho-p38 MAPK, and phospho-MAPKAPK-2. Enhanced chemiluminescence (ECL) detection was done according to the manufacturer’s guidelines (Lumi-Light®Plus, Roche Diagnostics, Mannheim, Germany).

Effects of RWJ 67657 on phosphorylation of p38 MAPK and its downstream substrate MAPKAPK-2 were measured after incubation of the cells with the p38 MAPK inhibitor and stimulation with TNFα and/or IL-1β for 30 minutes.

Statistics

One way analysis of variance with Dunnett’s post test or Bonferroni’s multiple comparison test was employed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, California, USA).

RESULTS

Effect of RWJ-67657 on protein production by rheumatoid synovial fibroblasts

Figure 1 shows the results of the production of MMP-1, MMP-3, TIMP-1, IL-6, and IL-8 by RSF (n = 5) after stimulation with TNFα and/or IL-1β, and also after pretreatment with RWJ 67657. Production of MMP-3 after stimulation with IL-1β without RWJ 67657 led to higher production than stimulation with TNFα (15.0-fold), and the same was true to a lesser extent with RWJ 67657 (11.0-fold, 1.2-fold). TIMP-1 production was not induced when stimulated with either cytokine. Stimulation with both cytokines had a synergistic effect on MMP-3 production: 22.7-fold compared to TNFα alone and 1.6-fold compared to IL-1β alone. Preincubation with RWJ 67657 resulted in a significant dose dependent decrease in MMP-3 production when cells were stimulated with IL-1β alone or together with TNFα. Only a high concentration (10 µM) of the p38 MAPK inhibitor had an effect on MMP-1 production, while for TIMP-1 there was no effect of p38 MAPK treatment.

Stimulation with IL-1β led to higher productions of IL-6 and IL-8 than stimulation with TNFα: 24.0-fold for IL-6 and 14.3-fold for IL-8. A dose dependent decrease in IL-6 and IL-8 production was seen after pretreatment with RWJ 67657.

We calculated the average percentage inhibition caused by treatment with RWJ 67657. More than 50% inhibition of MMP-3 and IL-8 production could be achieved at 1 µM, more than 50% inhibition of MMP-1 at 10 µM, and more than 50% inhibition of IL-6 production at 0.1 µM. Control experiments were done by adding 0.1% DMSO (at a concentration of 10 µM RWJ 67657) to stimulated RSF (n = 3). No significant
inhibition of protein production could be detected as a result of the DMSO (data not shown).

**Effect of RWJ 67657 on mRNA expression**

A time course study after stimulation with TNF-α and/or IL-1β was carried out to determine the time required for optimal mRNA expression. MMP-1 and MMP-3 mRNA expression was maximal after 24 hours, while IL-6, IL-8, and COX-2 mRNA had already reached maximum expression after six hours (data not shown).

Figure 2 shows the mean levels (expressed as -fold induction compared with untreated cells = 1) for MMP-1, MMP-3, TIMP-1, and ADAMTS-4 mRNA expression of three different RSF after six and 24 hours of stimulation and after pretreatment with RWJ 67657. As with protein production, mRNA expression of MMP-3 was much greater after stimulation with IL-1β than after stimulation with TNF-α: 40.1-fold after six hours of stimulation, and up to 149.4-fold after 24 hours. The equivalent values for MMP-1 mRNA expression were 3.4-fold after six hours and 2.1-fold after 24 hours, although the absolute expression increased with time. TIMP-1 mRNA expression hardly increased after stimulation (maximum two- to threefold compared with unstimulated cells). ADAMTS-4 mRNA expression could be measured in synovial fibroblasts and increased with time. Again the expression after IL-1β stimulation was greater than after...
**DISCUSSION**

In this study we showed significant inhibition by the p38 MAPK inhibitor RWJ 67657 of proinflammatory mediator and protease production in rheumatoid synovial fibroblasts. When inhibition was seen at the protein level, there was also inhibition at the level of mRNA expression, which means that this inhibition is at least at the level of RNA transcription. TNFα and IL-1 are considered the most important cytokines in the process of inflammation in rheumatoid arthritis. Studies in experimental models have shown that TNFα is indeed a pivotal cytokine in acute joint swelling, whereas IL-1β is the dominant cartilage destroying cytokine.21 Therefore we used both cytokines for activation of synovial fibroblasts to investigate the effects of a p38 MAPK inhibitor.

Stimulation of RSF with IL-1β or TNFα had different effects. Production of MMP-3 was greater after stimulation with IL-1β than with TNFα, although there was a synergistic effect of IL-6, IL-8, and COX-2 after stimulation for six hours and after treatment of three different rheumatoid synovial fibroblasts with different concentrations of RWJ 67657. Again there was a difference in expression after stimulation with TNFα or IL-1β. For IL-6, IL-8, and COX-2, respectively, stimulation with IL-1β gave 38.0-fold, 21.1-fold, and 18.3-fold higher expression than after stimulation with TNFα.

Significant inhibition of IL-6 mRNA expression was already seen at 0.01 μM RWJ 67657 when RSF were stimulated with IL-1β alone or together with TNFα. Inhibition of IL-8 mRNA expression was not significant, possibly because of a large interindividual response in IL-8 expression. COX-2 mRNA expression was significantly inhibited at 0.01 μM RWJ 67657 when the cells were stimulated with IL-1β or the IL-1β+TNFα combination.

To determine whether RWJ 67657 also affected cells that were already stimulated, 0.01 μM and 1 μM of p38 MAPK inhibitor were added before and one hour after TNFα+IL-1β stimulation of two RSF cultures, and IL-6 and COX-2 mRNA expression was analysed. One hour after stimulation, phosphorylation of p38 MAPK had already reached maximum. When RWJ 67657 was added one hour before stimulation, the decrease in mRNA expression with RWJ 67657 concentrations of 0.01 μM and 1 μM was 59.8% and 97.9%, respectively, for COX-2, and 38.4% and 71.5% for IL-6. When RWJ 67657 was added one hour after stimulation these values were 57.1% and 95.2% for COX-2 and 45.2% and 81.0% for IL-6. This shows that the p38 MAPK inhibitor also inhibited inflammatory mediator production in previously activated rheumatoid synovial cells.

Control experiments were carried out by adding 0.1% DMSO to stimulated RSF (RWJ 67657 concentration 10 μM). Significant reduction by 0.1% DMSO was seen only after IL-1β induced IL-6 mRNA expression and TNFα+IL-1β induced COX-2 mRNA expression. However, in both cases a significant reduction in mRNA expression was already found with 0.01 μM RWJ 67657 at non-inhibiting DMSO concentrations.

**Effect of RWJ 67657 on phosphorylation**

First we investigated the phosphorylation rate of p38 MAPK after stimulation with TNFα and/or IL-1β in rheumatoid synovial fibroblasts. As shown in Fig 4A, phosphorylation occurred rapidly and started after five minutes, reaching its maximum at 15 to 30 minutes for both stimuli. As expected, no inhibition of phosphorylation of p38 MAPK by RWJ 67657 was found (fig 4B). For MAPKAPK-2, which is a direct downstream substrate of p38 MAPK, no inhibition of phosphorylation of p38 MAPK by RWJ 67657 was found (fig 4B). For MAPKAPK-2, which is a direct downstream substrate of p38 MAPK, no inhibition of phosphorylation of p38 MAPK by RWJ 67657 was found (fig 4B). For MAPKAPK-2, which is a direct downstream substrate of p38 MAPK, no inhibition of phosphorylation of p38 MAPK by RWJ 67657 was found (fig 4B). For MAPKAPK-2, which is a direct downstream substrate of p38 MAPK, no inhibition of phosphorylation of p38 MAPK by RWJ 67657 was found (fig 4B).
Significant inhibition of induced production was seen when the cells were pretreated with 1 mM of the p38 MAPK inhibitor. MMP-1 protein production could be induced after stimulation (five- to sevenfold), but relevant inhibition was seen only at a concentration of 10 mM of RWJ 67657, which is too high for use in humans. A study by Parasrampuria et al. showed that a single oral dose of 0.25 to 30 mg/kg resulted in plasma concentrations of RWJ 67657 between 0.01 μM and 6 μM. No up- or downregulation was seen for TIMP-1 production after stimulation and treatment with RWJ. TIMP-1 is constitutively expressed, and the expression is not influenced by TNF-α or IL-1β, and consequently not by p38 MAPK inhibition either.

With quantitative real time RT-PCR we measured mRNA levels of MMP-1, MMP-3, TIMP-1, and ADAMTS-4. IL-1β induced higher levels of mRNA expression for MMP-1, MMP-3, and ADAMTS-4 than TNF-α. Moreover, we showed inhibition of mRNA expression for these genes by the p38 MAPK inhibitor. Others have found that activation of p38 MAPK in human skin fibroblasts enhances MMP-1 and MMP-3 expression by mRNA stabilisation. This is in agreement with our findings, indicating that inhibiting the p38 MAPK signal transduction route in RSF decreased expression of MMP-3 mRNA and to a lesser extent of MMP-1 mRNA. Work from our group has established the importance of MMP-3 as an indicator of radiological progression in early rheumatoid arthritis, especially of joint space narrowing, which represents cartilage degradation.

The expression of TIMP-1 mRNA was only affected after 24 hours of stimulation with increasing concentrations of RWJ 67657, which could have a protective effect by neutralising MMPs. As GAPDH levels were constant, possible adverse effects of RWJ 67657 did not induce this phenomenon.

IL-6 and IL-8 are important cytokines in inflammation and both are present at high concentrations in synovial fluids of patients with rheumatoid arthritis. Strong induction of both cytokines in RSF could be demonstrated after IL-1β stimulation particularly. RWJ 67657 significantly inhibited this induced IL-6 and IL-8 production at 0.01 μM and 0.1 μM. Suzuki et al. reported the decrease in IL-6 and IL-8 protein production after treatment with SB 203580, but no effect on mRNA expression, measured by traditional RT-PCR at a concentration of 30 μM. With quantitative real time RT-PCR we detected inhibition of both IL-6 and IL-8 mRNA expression. Therefore this study showed that IL-6 and IL-8 are inhibited by RWJ 67657 both at the protein and the mRNA level.

In 1998 Guan et al. reported that the induction of COX-2 and production of PGE2 were directly linked to activation of MEKK1 and consequently to activation of p38 MAPK. Recently it was reported that COX-2 mRNA stability is under regulation by p38 MAPK. In our study we demonstrated upregulation of COX-2 mRNA after stimulation with IL-1β or TNF-α, and very strong inhibition by RWJ 67657, especially after IL-1β induced expression. Effects of p38 MAPK inhibition are partly mediated through its downstream kinase MAPKAPK-2 and may involve phosphorylation of hsp27. Our results showed that after 30 minutes p38 MAPK was already maximally phosphorylated and that MAPKAPK-2 phosphorylation was blocked at a concentration of 0.1 μM. Addition of RWJ 67657 to stimulated cells did not affect the inhibitory capacities of the compound, so inhibition of the p38 MAPK signal transduction route in activated cells is possible.
p38 MAPK inhibitors have very potent effects on TNFα production by LPS stimulated monocytes at low concentration. For RWJ 67657 this was established at 3 nM.14 For monocyte derived macrophages, 50% inhibition was reached at a concentration of 30 nM.15 Our study here clearly showed that IL-1β is a stronger inducer of expression of inflammatory mediators by synovial fibroblasts than TNFα and may as such be the major cartilage destructive cytokine. p38 MAPK inhibitors such as RWJ 67657 inhibit both IL-1β and TNFα, as well as the responses induced by these cytokines. This dual activity of p38 MAPK inhibitors may be of major importance in the treatment of rheumatoid arthritis and other inflammatory conditions.

p38 MAPK inhibitors have effects on different cell types, which could enhance the therapeutic effects but also increase the risk of side effects. In the past, clinical trials with other p38 MAPK inhibitors have been stopped because of safety issues. One of the reasons for undesirable effects might be cross reactivity against other kinases, which was not the case for RWJ 67657. Furthermore we excluded induction of apoptosis in RSF following incubation with RWJ 67657 by staining cells with Annexin V and propidium iodide as described previously16 (data not shown). However, RWJ 67657 has been found to have acceptable safety and pharmacokinetic characteristics, warranting further investigation.17 There were no adverse effects associated with single doses of this drug. While the preliminary pharmacokinetic data suggest a twice daily dosing regimen, our data show significant effects at low concentrations. More research upon the effects of p38 MAPK inhibition on other cell types involved in inflammation will establish its applicability as a drug in the near future. The results presented in this study are promising.

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We gratefully acknowledge Dr Fred Breukelman and Dr Lex Boerboom for delivery of the synovial tissues. We thank Dr Marco Hammsen and Dr Sigga Asgeirsdottir for assistance with the PCR experiments and Dr Miek van Leeuwen for critically reading of the manuscript. We are grateful to Mrs Berber Doornbos-van der Meer for her excellent technical assistance. The work was supported by the Dutch Rheumatology Foundation and Johnson and Johnson Pharmaceutical Research and Development, Ranirian, New York, USA.

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doi: 10.1136/ard.2003.013011