Intramuscular gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane

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Abstract

Objectives—Cytokines, released from mononuclear cells (MNC) are mediators of joint destruction in rheumatoid arthritis (RA). The mechanisms of action of gold salts used in the treatment of RA are unknown. The aim of this study was to investigate cytokine expression and intensity of MNC infiltrate in the RA synovial membrane (SM) following treatment with sodium aurothiomalate (SAT).

Methods—Sequential blind needle biopsies were obtained at entry into the study and at two and 12 weeks after the start of SAT therapy in 10 patients with active RA. SMs were stained with a panel of monoclonal antibodies to assess cytokine expression (IL-1α, IL-1β, TNF-α, IL-6, and GM-CSF).

Results—There was a significant decrease in IL-1α, IL-1β, IL-6 and TNF-α expression 12 weeks after treatment (p < 0.004, p < 0.002, p < 0.009 and p < 0.004 respectively). This was noted in the lining layer, the perivascular aggregates and the connective tissue areas. Detailed examination of the MNC infiltrate showed a significant reduction in inflammatory monocytes (MONO) in the lining layer at two weeks (p < 0.03). A decrease in the number of CD68+ macrophages (MAC) was noted in the perivascular and connective tissue areas at 12 weeks. No significant changes were observed in the number of T and B cells and blood vessels.

Conclusion—The results suggest that gold may suppress RA disease activity by diminishing MONO and MAC numbers and consequently monokine production in the SM.

Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disease of unknown aetiology. Its pathogenesis is predicated on T cell and MONO/MAC activation with the release of a number of monokines such as interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α), which secondarily lead to the release of cytokines from other cells such as synoviocytes. Collectively, the release of these and other inflammatory mediators as well as the activation of synoviocytes, MONOs/MACs, chondrocytes and osteoclasts bring about the inflammatory and destructive changes which are characteristic of the disease. A crucial role for these pathogenic events is the accumulation of MONOs from the circulation. Bone marrow grafting experiments in mice have shown that the resident type A cells of the synovial lining layer are of bone marrow origin. After entry into tissues, MONOs mature into MACs and the differentiation steps involved have been studied in vitro and followed both in vivo and in vitro by appropriate monoclonal antibodies (mAb). Sorg et al have developed a series of monoclonal antibodies which mark the early acute inflammatory MONO (27E10) and the anti-inflammatory MAC (RM 3/1). There is increasing evidence that the function of MONO/MAC is dependent on the stage of differentiation reached by these cells. Thus MONO are potent producers of pro-inflammatory monokines such as IL-1, whereas optimal production of the IL-1 receptor antagonist (IL-1ra) requires that the MONO be matured to MAC by in vitro culture in the presence of immobilised IgG, as a form of model immune complexes, and granulocyte/macrophage colony stimulating factor (GM-CSF). Since inflammatory events may be regulated by the balance between pro-inflammatory cytokines, such as IL-1, and soluble inhibitors, such as IL-1ra, the shift of this balance to the latter could offer therapeutic benefits to the patient. Indeed, IL-1ra has been shown to be effective treatment for experimental models of arthritis.

The treatment of RA with SAT is still widespread despite a variable therapeutic response, serious side effects and lack of knowledge of its mode of action. Little attention has been paid to the changes which occur in the RA SM following the introduction of SAT. In previous studies, a reduction in the T-cell infiltrate was noted but only after six months of treatment by which time therapeutic benefit is well established. Other measurements of inflammatory capacity, such as decreased HLA-DR expression, decreased thickness of the synovial lining layer and reduction in the number of blood vessels, have proved equally disappointing.

So called disease modifying drugs (DMARDs), such as SAT, have been used for the treatment of RA on empirical grounds;
consequently, their mode of action is still uncertain and is based mainly on in vitro work. This is particularly true for SAT for which such diverse activities as inhibition of MONO accessory function in T lymphocyte activation, inhibition of production of rheumatoid factor production, interference with the antigen presenting function of HLA molecules and inhibition of endothelial cell proliferation, have been described, however, little or no evidence has been produced on the relevance of these findings to its in vivo mode of action. Recently, it has been shown that SAT will accelerate the in vitro maturation of MONO to MAC with a concomitant decrease in IL-1 production. Therefore, there is now the possibility to relate the therapeutic benefit with changes in expression of cytokines and the relative contribution of inflammatory MONO and downregulatory MAC within the synovial cell populations.

We reasoned that if SAT could induce maturation of MONO in the RA SM than there would be decreased production of pro-inflammatory cytokines as well as a decrease in the numbers of 27E10 positive monocytes, the former being dependent on the latter. In this study, multiple synovial biopsies were obtained from the knee joints of patients with RA before and at two and 12 weeks following the administration of SAT. Serial changes in cellular composition, with particular reference to MONO and MAC cells, and cytokine expression were examined using immunohistochemistry with appropriate mAb directed against cellular markers and against the inflammatory cytokines IL-1, TNF-α, GM-CSF and interleukin 6 (IL-6).

Patients and methods

PATIENTS AND TREATMENT PROTOCOL

Ten patients (table 1) with RA attending the Rheumatology Unit of Guy’s and Lewisham Hospitals were selected for a study comparing the clinical and synovial histological changes observed after the administration of intramuscular SAT or a combination of intramuscular SAT and methylprednisolone. All patients received intramuscular SAT which was given as a 10 mg test dose followed by 50 mg weekly until a total dose of 1-0 g was reached after which SAT was continued at 50 mg monthly. Five patients received 120 mg of methylprednisolone acetate intramuscularly at 0, 4 and 8 weeks of SAT whilst the other five received intramuscular 1 ml normal saline at the same time. Allocation between the two treatment groups was random. Patients were entered into the study if they required DMARDs and had either persistent synovitis despite non-steroidal anti-inflammatory drugs (NSAIDs) for three months or progressive erosions on radiographs and all had Westergren erythrocyte sedimentation rate (ESR) greater than 40 mm/h. All patients had an obvious knee synovitis. Patients were excluded if they had received previous treatment with gold or were aged less than 16 years or greater than 80 years. Proteinuria, glucocorticoid treatment, (oral or intramuscular) within three months of entry, were further exclusion criteria.

All patients gave their written consent and the study had the approval of the Lewisham and North Southwark District Ethics Committee.

CLINICAL AND LABORATORY ASSESSMENT OF DISEASE ACTIVITY

Patients were assessed by one rheumatologist at entry into the study and at two and 12 weeks following the administration of SAT. Routine measurements of disease activity included Ritchie articular index, visual analogue scale for pain (VAS), grip strength (using a folded sphygmomanometer cuff inflated to 30 mm/Hg, three attempts and results expressed as the mean value of both hands). Laboratory assessments included full blood count, ESR in the first hour and rheumatoid factor.

SYNOVIAL BIOPSY

Synovial samples were obtained from one knee joint of all patients by a blind biopsy technique at 0, 2 and 12 weeks using a Williamson-Holt synovial biopsy needle (Shrimpton and Fletcher Needle Industries, Redditch, Worcestershire, UK) under sterile conditions. Where only one knee was involved, the specimens were obtained from the affected side, when both knees were involved, the biopsy was performed on a side selected at random. Repeat biopsies were performed on the same knee joint. Local anaesthesia using 2% lignocaine was administered. To facilitate the biopsy procedure, 20 ml of sterile saline were instilled into each joint. Multiple synovial samples (minimum of four) were obtained from the supra-patellar pouch of the knee joint from as wide an area as possible within reach of the needle.

TISSUE PREPARATION AND STAINING

Synovial specimens were snap frozen within 30 minutes of the biopsy procedure after orientation in optimal temperature cutting compound (OTC) in isopentane cooled in liquid nitrogen. Samples were stored at −70°C until sectioned for immunohistochemical staining. Frozen samples were embedded in OCT and 5 μm sections were cut with a cryostat at −20°C. Sequential sections were mounted on gelatine/poly-l-lysine coated slides and dried overnight at room temperature. Sections were fixed in acetone for 20 minutes wrapped in tin foil and stored at −70°C until further use. Cytokines and cellular antigens were determined by an indirect immunoperoxidase technique. The mAb

<table>
<thead>
<tr>
<th>Table 1 Characteristics of the patients studied</th>
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<tbody>
<tr>
<td>Total number</td>
</tr>
<tr>
<td>Male:Female</td>
</tr>
<tr>
<td>Mean (SD) age (range)</td>
</tr>
<tr>
<td>Mean (SD) disease duration (range)</td>
</tr>
<tr>
<td>IgM-RF positive (latex)</td>
</tr>
<tr>
<td>Nodules</td>
</tr>
</tbody>
</table>
Cytokine expression and macrophage numbers in the rheumatoid synovial membrane

CONFIRMATION OF CYTOKINE IMMUNOREACTIVITY

The specificity of the cytokine monoclonal antibodies has been extensively studied and demonstrated by the providers of these proteins. Anti-TNF-α (personal communication from MA Cousin, Roussel) was obtained after fusion of immunised BALB/c spleen cells with SP2/0-Ag14 myeloma cells. Partially purified murine immunoglobulins (sodium sulphate precipitated from ascitic fluid dissolved and dialysed in PBS) was used. This antibody did bind to r-Hu-TNF-α coated on ELISA plates and neutralised r-Hu-TNF-α cytopoictic activity an L929 cells. The specificity of the other cytokine monoclonal antibodies has been documented in published manuscripts: IL-1α, IL-1β, GM-CSF and IL-6.

MICROSCOPIC EVALUATION

Evaluation was undertaken in a minimum of four different synovial tissue samples for each patient at each time point. Cytokine expression and intensity of cellular infiltrate were assessed in three areas of the synovial membrane: (1) lining layer, (2) perivascular lymphoid aggregate, where there was a cellular infiltrate of at least five cells in depth surrounding a central vessel, (3) connective tissue, where mononuclear cells were randomly arranged throughout without the formation of discrete perivascular aggregates.

All microscopic evaluations were performed by one observer (NP) who was ‘blind’ to the names of the patients and time of biopsy. Furthermore, to rule out intra-observer variation, samples from five patients were read on three different occasions. No statistically significant differences were observed between the three readings. Samples were also assessed by another observer (GY); the results agreed with the first observer.

CYTOKINE STAINING

Cytokine expression was assessed by estimating the intensity of staining and the number of the positively staining cells in the three areas defined above in a minimum of four biopsy samples at each time point for each patient. The intensity of staining was estimated by devising a visual analogue scale (VAS) from 0–15 cm, where 0 indicated no staining at all and 15 maximal staining. All sections were examined and results were expressed as a mean. For the number of positively staining cells, a total of 200 cells was examined in each of the areas and the number of positively staining cells was expressed as a percentage. Positivity was noted when complete staining of the cell was observed.

MONONUCLEAR CELLULAR INFILTRATE

The number of cells staining positively for a particular cell marker was counted out of a total of 200 cells in each of the lining layer, perivascular and connective tissue areas and the results expressed as a percentage.

SYNOVIAL LINING LAYER THICKNESS

Synovial lining layer thickness was estimated by assessing all visible lining layer at a magnification of ×400. The thickness was quantified according to the cell depth. Three random readings per high power field (HPF) were recorded and the results expressed as a mean.
VASPAIN
Ritchie index
Grip (g%)
Haemoglobin (mm/hr)
Results
Not present
CD22+
Not
CD68+
10-9
Others
Table 3
Mononuclear cellular infiltrate, number of blood vessels and lining layer thickness of the synovial membrane at entry, two and 12 weeks of the study

<table>
<thead>
<tr>
<th>Mononuclear cellular infiltrate</th>
<th>Lining layer</th>
<th>Perivascular</th>
<th>Connective tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>CD1+</td>
<td>Not present</td>
<td>39.5 (24.3)</td>
<td>37.8 (28.7)</td>
</tr>
<tr>
<td>CD4+</td>
<td>Not present</td>
<td>18.6 (19.6)</td>
<td>28.4 (19.1)</td>
</tr>
<tr>
<td>CD8+</td>
<td>Not present</td>
<td>24.7 (12.2)</td>
<td>27.5 (21.2)</td>
</tr>
<tr>
<td>CD22+</td>
<td>Not present</td>
<td>91.9 (9.5)</td>
<td>12.7 (10.6)</td>
</tr>
<tr>
<td>2TE10+</td>
<td>18.9 (13.0)</td>
<td>12.2 (9.2)*</td>
<td>9.1 (9.0)*</td>
</tr>
<tr>
<td>CD14+</td>
<td>10.9 (6.8)</td>
<td>17.2 (11.6)</td>
<td>12.6 (8.5)</td>
</tr>
<tr>
<td>RM1/3</td>
<td>17.4 (10.3)</td>
<td>19.2 (23.7)</td>
<td>12.1 (7.1)</td>
</tr>
<tr>
<td>CD68+</td>
<td>53.3 (32.0)</td>
<td>61.0 (19.0)</td>
<td>65.9 (11.5)</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>65.9 (11.5)</td>
<td>65.9 (11.5)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62.3 (18.8)</td>
<td>45.8 (24.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18.9 (26.4)</td>
<td>38.0 (21.5)</td>
</tr>
</tbody>
</table>

Results expressed as mean (SD). NS = non-significant. P values, differences between week 0 and week 12.

Table 2
Assessment of disease activity at entry, two weeks and 12 weeks of the study

<table>
<thead>
<tr>
<th></th>
<th>Entry</th>
<th>2 weeks</th>
<th>12 weeks</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAS pain (cm)</td>
<td>6-6 (1-0)</td>
<td>6-2 (1-2)</td>
<td>4-5 (0-8)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Ritchie index</td>
<td>8-6 (1-1)</td>
<td>8-0 (1-6)</td>
<td>4-7 (3-1)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Grip strength (mmHg)</td>
<td>204 (99)</td>
<td>303 (81-0)</td>
<td>344.0 (78-0)</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Haemoglobin (g%)</td>
<td>13-1 (2-3)</td>
<td>13-0 (1-9)</td>
<td>12-9 (1-8)</td>
<td>NS</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>43-3 (33-0)</td>
<td>38-0 (27-0)</td>
<td>37-4 (32-0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results expressed as mean (SD). NS = non-significant. P values, differences between week 0 and week 12.

CELLULAR COMPOSITION
Examination of the cellular infiltrate revealed changes in the MONO/MAC population only. CD68+ cells were the most commonly identified MAC in the three areas of the synovium examined. The numbers of 2TE10+, CD14+ and RM3/1+ MONO/MAC were similar. There was a significant decrease in the number of 2TE10 positively staining MONO from 18-9% at entry to 12-2% at two weeks and to 9-1% at 12 weeks in the lining layer (p < 0.04 and p < 0.03 respectively), table 3. There was a significant reduction in the number of CD68 positively staining MACs from 62-3% at entry to 44-1% at 12 weeks in the perivascular areas and from 49-5% at entry to 38-5% at 12 weeks in the connective tissue areas (p < 0.02 and p < 0.008 respectively), table 3. No changes were observed in the percentage of positively staining CD14 and RM3/1 cells. The numbers of CD3+, CD4+, CD8+ and CD22+ cells did not alter.

The number of blood vessels and synovial lining layer thickness did not vary significantly throughout the study period.

CYTOKINE EXPRESSION
Cytokine expression was estimated by assessing the intensity of staining and by counting the number of positively staining cells. Significant changes were noted, in all areas of the synovium, 12 weeks after the start of therapy compared with entry into the study (fig 1a–d). No changes were noted at two weeks (data not shown).

In the lining layer (fig 2), there was a significant decrease in the intensity of staining for IL-1α from 5-0 at entry to 4-2 at 12 weeks (p < 0.05), IL-1β from 6-0 to 2-5 (p < 0.006) and IL-6 from 4-2 to 3-8 (p < 0.05). TNF-α fell from 4-3 to 2-0 but this just failed to reach statistical significance (p < 0.064). There was no significant change in the percentage of positively staining cells.

In the perivascular areas (fig 3), there was a significant decrease in the intensity of staining for IL-1α from 6-8 to 5-2 (p < 0.01), IL-1β from 4-1 to 2 (p < 0.01), TNF-α from 3-5 to 1-2 (p < 0.05) and IL-6 from 3-5 to 1-5 (p < 0.01) (fig 3). Furthermore, there was a significant reduction in the number of IL-1α and IL-6 positively staining cells, from 12% to 0% = before treatment, 2 = two weeks after treatment, 12 = 12 weeks after treatment.
HPF = high power field, * = significant values, results expressed as mean (SD).
Cytokine expression and macrophage numbers in the rheumatoid synovial membrane

Figure 1. (A) Interleukin-1α staining in the synovial membrane at entry into the study (magnification ×650); (B) Interleukin-1α staining in the synovial membrane 12 weeks after starting sodium aurothiomalate therapy (magnification ×650); (C) Interleukin-1β staining in the synovial membrane at entry into the study (magnification ×650); (D) Interleukin-1β staining in the synovial membrane 12 weeks after starting sodium aurothiomalate therapy (magnification ×650); (E) Tumour necrosis factor-α staining in the synovial membrane at entry into the study (magnification ×650); (F) Tumour necrosis factor-α staining in the synovial membrane 12 weeks after starting sodium aurothiomalate therapy (magnification ×650).

5% (p < 0.04) and from 5% to 0 (p < 0.02) respectively (fig 3).

In the connective tissue area (fig 4), there was a significant decrease in the intensity of staining for IL-1α from 5.2 to 2.8 (p < 0.02), IL-1β from 3.9 to 1.5 (p < 0.004), TNF-α from 3.9 to 1 (p < 0.02), IL-6 from 3.8 to 1.8 (p < 0.05) and GM-CSF from 3.5 to 1.8 (p < 0.05). Furthermore, there was a significant reduction in the number of IL-1α, and IL-6 positively staining cells, from 14% to 3% (p < 0.04) and from 6% to 2% (p < 0.04) respectively.

Discussion
Ten patients with active RA were studied prospectively for 12 weeks after the introduction of SAT or a combination of SAT and intramuscular steroid therapy. SM cytokine expression and intensity of MNC infiltrate were examined at 0, 2 and 12 weeks. Clinical improvement was noted in all patients at 12 weeks.

Monokine expression (IL-1α, IL-1β, TNF-α and IL-6) was diminished at 12 weeks in most areas of the synovium. There was a significant decrease in the number of 27E10+ MONOs at two and 12 weeks in the lining layer. CD68+ MACs were reduced at 12 weeks in the perivascular and connective tissue areas. CD14+ MONOs remained unchanged. No significant differences were noted between the patients receiving SAT and a combination of SAT and methylprednisolone in the SM cytokine staining and intensity of MNC infiltrate. These findings may suggest a lack of sensitivity of the methods used or that the early clinical effects of methylprednisolone may be due to effects on cells or cytokines not examined in the present study.

This is the first study to examine cytokine expression in the rheumatoid synovium following treatment. Our results suggest that SAT may alter MONO/MAC function by inhibiting monokine production in the SM and that this reduction may be responsible for the clinical improvement seen in these patients since they occurred at the same time at 12 weeks. In vitro studies show that the inhibitory effect of gold on lymphocyte proliferation is enhanced when MONO numbers are reduced.
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and reversed when untreated MONOs are added back. Gold has a direct effect on MONO functions which are acquired during differentiation and the ability of MONOs to spontaneously produce the second complement component (C2), to lyse chicken erythrocytes and to kill certain nucleated target cells. Gold inhibits the stimulation of MONO C2 production by γ-interferon and the markers of monocytic differentiation in HL-60 cells. Haynes et al. showed that several gold thiolates used in the treatment of RA inhibit the lymphoproliferative activity of human IL-1 as measured by the lymphocyte activating factor assay.

In clinical studies, levels of circulating IL-1β and IL-1α in patients with RA have been correlated with clinical disease activity and the levels of IL-6 with ESR, rheumatoid factor and CRP. A lower percentage of IL-1α positive cells was detected in the peripheral blood of patients receiving parenteral gold.

The description of a naturally occurring IL-1Ra suggests that the body mounts its own response to inflammation. IL-1β and IL-1ra are separately regulated during monocyte differentiation. IL-1β is produced by CD14+ MONOs and IL-1ra by human MONOs cultured on a substrate of immune complexes or adherent IgG. IL-1ra has been detected in RA synovial fluid and in SM by immunoperoxidase and in situ hybridisation techniques. IL-1ra competes with IL-1 for the occupancy of the IL-1 cell surface receptors.
but cannot trigger the cellular responses typical of IL-1. \textsuperscript{19} In addition, it blocks the production of IL-1 induced IL-1, TNF and IL-6 production from human peripheral blood mononuclear cells as well as from purified MONOs. \textsuperscript{40} By inhibiting IL-1 production, gold salts may enhance the IL-1 neutralising effects of IL-1ra.

Changes in the immunohistological features following the introduction of gold have received very little attention. Previous studies have demonstrated a reduction in the number of infiltrating T and B cells at six\textsuperscript{41} and 12 months. \textsuperscript{42} The clinical relevance of these findings is doubtful as improvement in disease activity occurs earlier. Neither of these studies examined the MACs in the SM. MACs play an important role in the initiation and maintenance of rheumatoid synovitis. They process and present antigens to T cells, can stimulate blood vessel formation\textsuperscript{43} and represent a steady-state population of cells moving from blood vessels to the synovial lining layer. CD14 molecules participate in the adhesion of MONOs to cytokine stimulated endothelial cells\textsuperscript{44} and migrate through the synovium to form the type A lining layer cells.\textsuperscript{45} Within inflammatory sites, such as the rheumatoid synovium, MONOs are stimulated by cytokines such as IL-1, TNF-\alpha and GM-CSF to differentiate into MACs,\textsuperscript{46} thus survival of MONOs is a critical component to the maturation process. In the absence of IL-1\textbeta, TNF-\alpha, GM-CSF and IFN-\gamma MONOs undergo programmed cell death or apoptosis.\textsuperscript{47} Gold has the capacity to selectively inhibit some MONO functions which are associated with MAC differentiation\textsuperscript{48,49} and has been shown to inhibit markers of monocytic differentiation in HL-60 cell lines. The unchanged numbers of CD14+ cells throughout the study period suggest continued entry of MONOs into the RA synovial membrane. The reduction in 27E10, a MONO marker of early acute inflammation, at two weeks may be a steroid related effect, but we did not record a rise in RM3/1+ cells which have been shown to increase in vitro following the addition of dexamethasone.\textsuperscript{4} The decrease in CD68+ MACs at 12 weeks may suggest a failure of maturation of MONOs to MACs as MONOs die by apoptosis if not stimulated by inflammatory cytokines such as IL-1, TNF-\alpha and GM-CSF.\textsuperscript{45} It is also possible that by decreasing cytokine production gold salts also reduce endothelial cell adhesiveness for leukocytes\textsuperscript{46} and further contribute to the decrease in MONO/MAC numbers within the synovium.

Thus a plausible scenario for the effects of gold salts can be constructed on the basis of these in vivo and in vitro observations. Before gold therapy, CD14+ MONOs enter the rheumatoid synovium by binding to endothelial cells. In the joint, MONOs are activated and release monokines responsible for maintaining cell viability and promoting the maturation of MONOs to MACs. In the presence of gold salts there is a decrease in the endothelial cell adhesiveness resulting in a reduction of MONO entry into the RA synovium and reduction in cytokine production. In the absence of these monokines MONO viability is not maintained and MAC numbers decrease. In addition, gold accelerates the maturation of the remaining MONOs to MACs. These mature MACs may release IL-1ra and other unidentified regulators of inflammation which in turn downregulate the inflammatory response by inhibiting cytokine release.

In conclusion, the findings in this study suggest that SAT may interfere with the mechanisms responsible for persistent rheumatoid synovitis. MONOs may be an important source of inflammatory cytokines which are relevant to joint destruction. Further studies are needed to determine the precise role of these cells in the joint inflammation, and to identify the mechanisms by which gold reduces their effects.

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