Increased Monocyte Chemoattractant Protein-1 in Knee Joints of Rats with Adjuvant-Induced Arthritis: *In Vivo* Microdialysis

SHAO-HSIANG LIU, CHIH-SHUNG WONG, and DEH-MING CHANG

ABSTRACT. ***Objective.*** Imbalance of inflammatory and antiinflammatory cytokines plays a critical role in the pathogenesis of rheumatoid arthritis (RA). Precise determination of these cytokines would lead to better understanding of the progression of RA.

Methods. We developed an *in vivo* microdialysis technique to directly monitor cytokine profiles in knee joints of rats with adjuvant-induced arthritis (AIA). Microdialysates drained from knee joints of rats with AIA and controls were collected and cytokine concentrations were measured by ELISA. Pathological changes of the knee joints and the source of monocyte chemoattractant factor–1 (MCP-1) secretion were also determined by histology and immunohistochemistry.

Results. MCP-1 expression in knee joints was significantly higher in AIA rats with erosive changes in their ankles than in normal rats, while interleukin 6 (IL-6) levels were similar in both cases. IL-1ß and interferon-γ were not detectable in the microdialysates. Increased synovial proliferation and mononuclear inflammatory infiltrates were observed. Synovial cells and mononuclear inflammatory cells expressed both MCP-1 and its receptor, CCR2.

Conclusion. Our results indicate that the *in vivo* microdialysis technique is capable of detecting cytokines in the knee joints of rats. Increased expression of MCP-1 and CCR2 in knee joints of AIA rats suggests a role for this cytokine in triggering the mechanisms involved in the pathogenesis of knee joint after ankle erosion. (J Rheumatol 2005;32:2205–11)

Key Indexing Terms:
RHEUMATOID ARTHRITIS — ADJUVANT-INDUCED ARTHRITIS
IN VIVO MICRODIALYSIS — MONOCYTE CHEMOATTRACTANT PROTEIN-1

Rheumatoid arthritis (RA) mainly affects diarthrodial joints, causing inflammation of the synovial tissue and adjacent cartilage and underlying bone. Generally small joints are affected before the larger ones. The imbalance of inflammatory and antiinflammatory cytokines plays a critical role in the pathogenesis of RA. Studies of the role of these cytokines are faced with a number of difficulties; the most important is to monitor cytokine dynamics in synovial tissue analyses at different stages of disease progression.

In vivo microdialysis is a technique that detects *in vivo* microenvironment mediator responses to physiological stimulation or a pathological situation. Originally developed for neuropharmacological research, this technique involves the insertion of a microdialysis probe into the tissue of interest[1-4]. Low molecular weight compounds diffuse through a fiber at a constant rate from the extracellular fluid into a physiological salt solution in the probe, and the fluid thus collected, called a microdialysate, is analyzed. Although it can be argued that the insertion of a microdialysis probe may damage the microenvironment and thus confound the subsequent analyses, the damage apparently is limited to a few cell layers adjacent to the probe. To understand RA disease progression, developing a way to monitor small molecules precisely, locally, and continuously in both the diseased and normal joint microenvironment has become an important technical goal.

Interleukin 1ß (IL-1ß) plays an important proinflammatory role in RA[5]. IL-6 influences various adhesion molecules and cytokines, and these mediators then may modulate cartilage destruction[6]. IL-1ß and IL-6 can further induce another important component, monocyte chemoattractant factor-1 (MCP-1), which leads to monocyte infiltration in RA[7,8]. The inflammation in RA is also associated with enhanced Th1 cell activity, and a decreased Th2 cell activity. Th1 cell activity is defined by interferon-γ (IFN-γ) production[9]. Since IL-1ß, IL-6, MCP-1, and IFN-γ are impor-

From the Graduate Institute of Life Science; Department of Rheumatology/Immunology/Allergy; and Department of Anesthesiology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan.

Supported by the National Science Council, Taiwan, Republic of China (NSC 91-2314-B-016-046).

S-H. Liu, MS, Graduate Institute of Life Science, Department of Rheumatology/Immunology/Allergy; C-S. Wong, MD, PhD, Department of Anesthesiology; D-M. Chang, MD, MS, FACR, Department of Rheumatology/Immunology/Allergy.

Address reprint requests to Dr. D-M. Chang, Rheumatology/Immunology/Allergy, Tri-Service General Hospital, 325 Cheng-Kung Road, Section 2, Neihu 114, Taipei, Taiwan. E-mail: ming0503@ms3.hinet.net

Accepted for publication June 29, 2005.

tant mediators in RA, these mediators were monitored using our *in vivo* microdialysis technique in this study.

MATERIALS AND METHODS

Construction of the microdialysis probe. The intraarticular microdialysis probe was constructed using two 2-inch polyethylene (PE-5) tubes (0.008 inch inner diameter, 0.014 inch outer diameter), 2 polycarbonate tubes (194 µm outer diameter, 102 µm inner diameter; 1 cm long), and a hollow cuprophan fiber (dialysis fiber/membrane; 1.5 cm long, 300 µm outer diameter, 200 µm inner diameter, 50 kDa molecular weight cutoff; DM-22, Eicom Co., Kyoto, Japan). A Nichrome-Formvar wire (0.0026 inch diameter, 4.8 inch long; A-M Systems Inc., Everret, WA, USA) was passed through the 2 PE-5 tubes, 2 polycarbonate tubes, and the cuprophan fiber. The 1 cm polycarbonate tube was covered halfway by a sleeve of PE-5 tube joined by a sleeve of the cuprophan fiber. The active dialysis region, about 0.5 cm long, was in the middle (Figure 1). Thin-layer epoxy glue was used to connect all parts of the dialysis probe. The probe was connected to a PE-10

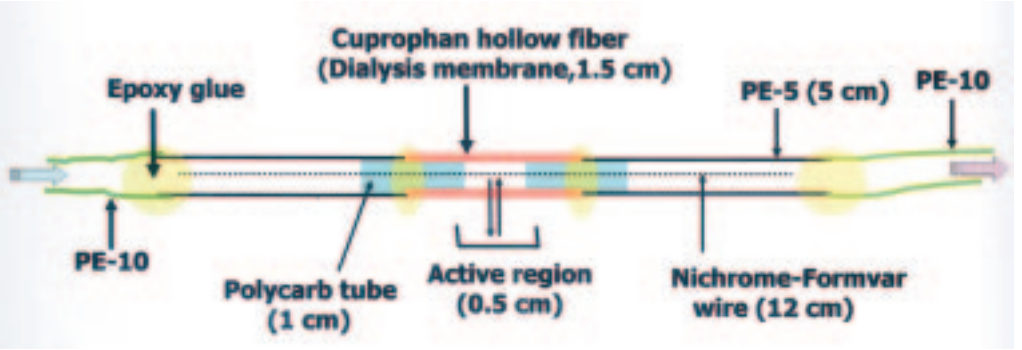
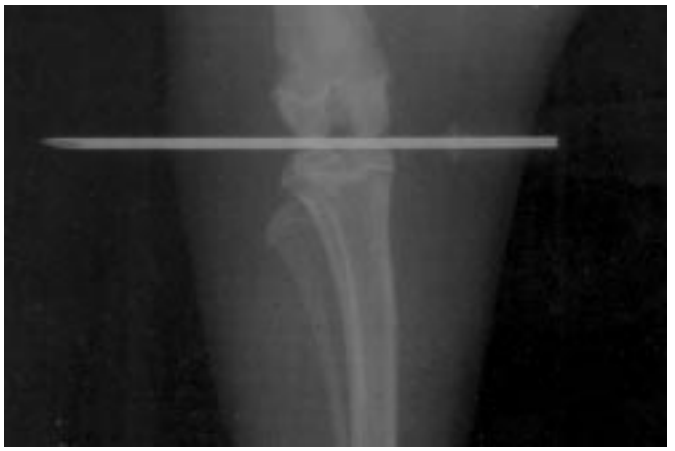
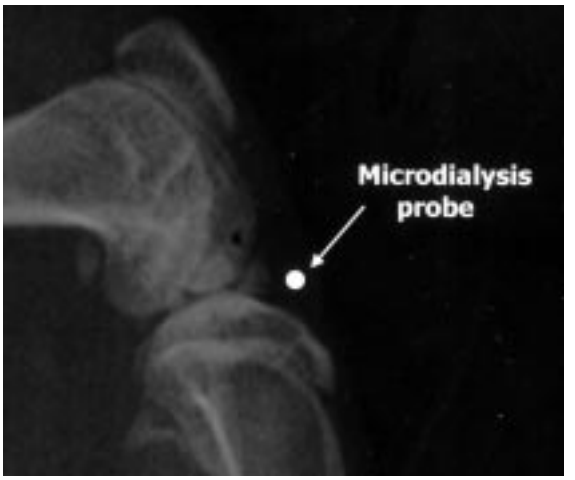


Figure 1. The microdialysis probe. The active region (0.5 cm) of the probe for joint space fluid dialysis is shown. One end of the probe was connected to a syringe pump. Ringer's solution perfusate was pumped through the device (left to right in the illustration); dialysate was collected directly into microtubes.





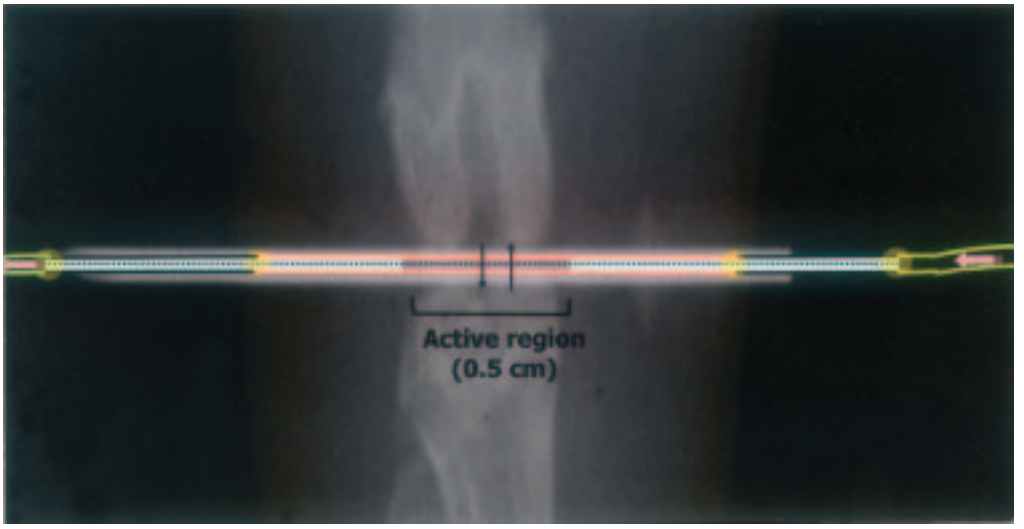


Figure 3. Radiographs of normal rat knee joint show location of the needle passed through the joint space. The microdialysis probe was inserted through the needle into the knee joint (lower panel); the needle was removed, leaving the active region of the probe inside the joint.

catheter (~30 cm) at one end, sealed to an external silastic tube connected to a syringe pump (CMA-100, CMA/Microdialysis Inc., Solna, Sweden) with silicone sealant. The cytokine expression pattern and profile in synovial fluid from rats with adjuvant-induced arthritis (AIA) were measured using this probe.

In vitro microdialysis assay. Relative recovery rate (R) was defined as the concentration (Cp) of a given substance in the microdialysis perfusate compared with its concentration (Cd) in the dialysate. Then the relative recovery rate was calculated as: R(%) = Cd/Cp.

During *in vitro* measurements, the recovery rate of the dialysis probe was determined at a perfusion rate of 1 µl/min. Glucose, a low molecular weight molecule, was added into the saline solution (10 mM). Then the glucose concentration in the dialysate was measured using a complete blood glucose monitoring system (Johnson & Johnson Co., Milpitas, CA, USA). IL-1ß R&D Systems, (Minneapolis, MN, USA), a high molecular weight molecule, was also diluted in saline solution (500 pg/ml) and its concentration in the dialysate was measured by ELISA.

Induction of arthritis. Male Lewis rats (250–300 g) were immunized at the base of the tail with 1 mg *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI, USA) in 0.1 ml of mineral oil on Day 0. Paw swelling was usually observed by Day 10–12, peak swelling by Day 14–16, and chronic inflammation thereafter. After 20–22 days, the ankle joint started to undergo bone erosion. Before starting microdialysis measurement, we determined that the rats were in the ankle erosion stage by observing clinical patterns and histological assessment. The paw thickness and knee joint width were measured with a caliper. After 35 days of induced arthritis, every arthritic rat had a swollen and irreversibly deformed ankle and was then subjected to the microdialysis experiment and histology.

Animal preparation and in vivo microdialysis procedure. The handling and use of rats for experimental purposes in this study conformed to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiology Society and by the National Defense Medical Center Animal Care and Use Committee. Rats were anesthetized by urethane (120 mg per 100 g rat body weight). The rat was placed on a heating blanket to maintain a core body temperature during anesthesia. The hindlimbs were then held in a partially fixed position and the microdialysis probes were inserted. The knee of each healthy control and AIA rat was shaved and disinfected with betadine solution before implanting with a probe. A 23-gauge needle was passed through the joint capsule lateral to the patellar ligament; the microdialysis probe was inserted from one end to the other through the needle into the knee joint, then the needle was removed. The microdialysis probe and silastic tube were connected by a PE-10 catheter as described. The end of the external silastic tube was connected to the syringe pump for perfusion. The sample was collected for 4 hours at the other end of each probe.

The dialysis system was perfused with Ringer's solution, consisting of 123 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 2 mM $MgSO_4$, 2 mM CaCl, and 10 mM dextrose, pH 7.4. The microdialysis probe was initially flushed for 30 min with Ringer's solution at a perfusion flow rate of 1 µl/min. Samples (microdialysates) were then collected in polypropylene tubes kept on ice and frozen at –80°C until assayed.

Determination of cytokines and chemokines. The frozen stocks of the microdialysate were thawed to room temperature before analysis. Cytokines and chemokines were detected using the ELISA method. All ELISA systems were purchased commercially (IL-1ß and IFN-γ from R&D Systems, Minneapolis, MN, USA; IL-6 and MCP-1 from Endogen, Rockford, IL, USA) and performed following the protocol from the manufacturer. According to the manufacturers' information, the ELISA range is 38–1500 pg/ml and the sensitivity is < 5 pg/ml.

Histological and radiological assessment of rat joints. Rats were sacrificed by CO_2 narcosis. Ankles were injected intraarticularly with 20 µl of 10% neutral buffered formalin and then removed, skinned, and fixed for 24 h in buffered formalin. Before paraffin embedding, the joints were decalcified in 10% EDTA for 1 day. Sections (5 µm thick) were stained with hematoxylin and eosin; all joints were radiographed.

Immunohistochemical analysis. Localization of MCP-1 and CCR2 was examined immunohistochemically by the avidin–biotin–peroxidase complex method, using a Vectastain ABC kit (Vector, Burlingame, CA, USA). Paraffin sections were placed on coated slides, deparaffinized, and washed in phosphate buffered saline (PBS). After inactivation of endogenous peroxidase with 0.5% citrate acid in PBS for 10 min, sections were preincubated with normal horse serum for 1 h. Then the sections were incubated with primary goat anti-rat MCP-1 (SC-1785) or goat anti-rat CCR2 antibodies (SC-7935; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, washed with PBS, and incubated with biotinylated anti-goat IgG (Vector) for 1 h. After washing, sections were reacted with a Vectastain ABC reagent. The sections were reacted with AEC (Vector) in PBS and counterstained with hematoxylin.

Data analysis. Data were analyzed using Student's t test. P values < 0.05 were considered significant.

RESULTS

Microdialysis probe and technique pretest. As the first group to detect cytokines in synovial fluid using this microdialysis technique, we had to demonstrate that our microdialysis probe and method (Figure 1) was functional and appropriate. The relative recovery of glucose was found to be approximately 42% (Figure 2A), and the relative recovery of IL-1ß was found to be approximately 17% (Figure 2B). The results suggested that the performance of our microdialysis technique was applicable and relative recovery

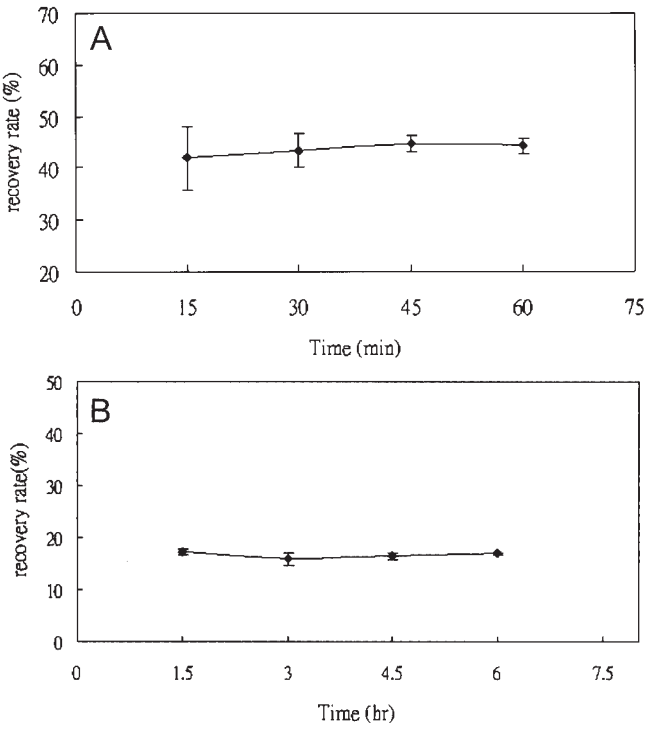


Figure 2. In vitro microdialysis assay. (A) Relative recovery rate (%) of glucose (molecular weight 180 Da) as a function of dialysis time. (B) Relative recovery rate (%) of IL-1ß (molecular weight 17 kDa). Perfusion rate was 1 µl/min (probes n = 6 per group).

measurements were consistent. However, the relative recovery rate was somewhat lower with higher molecular weight molecules.

Clinical patterns in AIA rats. Gross observations were carried out on every ankle and knee joint of controls and AIA rats. The paw thicknesses of control and AIA rats were 6.025 ± 0.33 mm versus 10.413 ± 1.08 mm, respectively ($p < 0.05$); the knee joint width was 9.466 ± 0.256 mm without a statistically significant difference between the 2 groups. The radiographs confirmed that arthritic rats were in the ankle erosion stage; however, the knee joints in both AIA rats and controls were radiologically normal (data not shown).

In vivo microdialysis. Originally, we designed the microdialysis probe technique to detect cytokines in ankle joints of AIA rats. Since the ankle joint was in a state of ankylosis, we could not enter the ankle joint. Instead, the knee joint synovial fluid was assayed first. We positioned the needle and located our microdialysis probe with the aid of radiographic monitoring (Figure 3) for clear visualization in the knee joint space and to confirm the probe did not perturb other tissues. Further, after collection of microdialysates, we sacrificed the rats and dissected the knee joint to determine that the microdialysis probe was at the correct location and was still functional.

As the microdialysis probe could potentially be obstructed by matrix components, cellular debris, and tissue mucus, we assayed MCP-1 concentrations in samples withdrawn at serial timepoints from normal Lewis rats (Figure 4). We found no significant differences in MCP-1 concentrations at 4 h and 8 h of dialysis time (140.9 ± 26.5 vs 147.2 ± 26.6 pg/ml, respectively; n = 6). Thus the microdialysis probe was not significantly obstructed, and its function was stable for at least 8 h.

Cytokine expression in knee joints of AIA rats in chronic inflammation stage. Comparison of *in vivo* cytokine patterns in knee joints of normal and arthritic rats revealed a difference in MCP-1 concentrations: 151 ± 50 pg/ml versus 250 ± 76 pg/ml, respectively ($p < 0.01$; n = 6), whereas there was no difference in IL-6 concentrations: control 84 ± 42 pg/ml versus AIA 84 ± 44 pg/ml (n = 6; Figure 5). Both IL-1ß and IFN-γ were not detectable. On the basis of our findings, tissue changes that occurred in grossly normal knee joints of AIA rats after 35 days of inflammation were evaluated. Surprisingly, histological staining of normal and AIA rat knee joints revealed both cell proliferation and mononuclear inflammatory infiltrates in the synovial tissue of AIA rats (data not shown). Further, immunohistochemistry analysis was performed to determine which cells expressed MCP-1 and its receptor, CCR2; the findings demonstrated that MCP-1 and CCR2 expression on synovial cells and mononuclear cells was significantly higher in AIA rats than in normal rats (Figure 6).

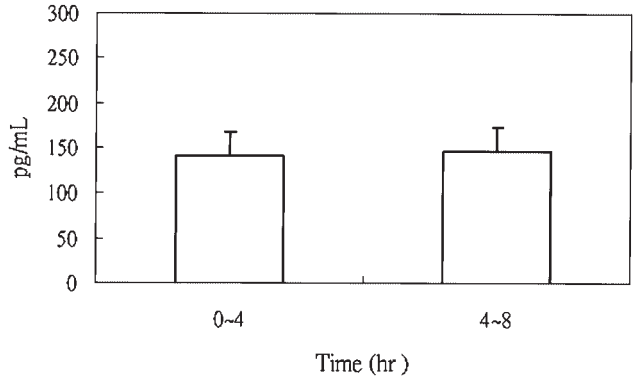


Figure 4. Levels of MCP-1 in knee joints of normal rats (n = 6). Microdialysates were collected for 4 and 8 hours after probe insertion and were analyzed by ELISA.

DISCUSSION

Although AIA is a well established model to study inflammatory arthritis in rats, it is difficult to study the dynamic biochemical changes in synovial fluid. The *in vivo* microdialysis technique, involving insertion of a probe into the knee joint of rats, provided a way to continuously monitor local cytokines from synovial fluid.

Development of the microdialysis probe design and its

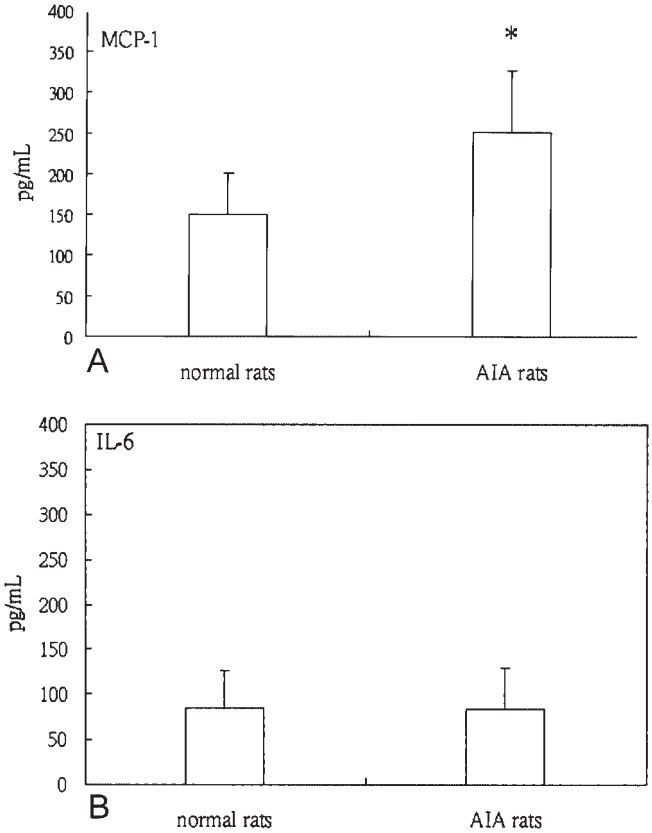


Figure 5. Cytokine microdialysate samples were collected for 4 hours and measured by ELISA. MCP-1 (A) but not IL-6 (B) secretion differed in knee joints between normal and AIA rats with erosive ankle disease (*$p < 0.01$; n = 6).

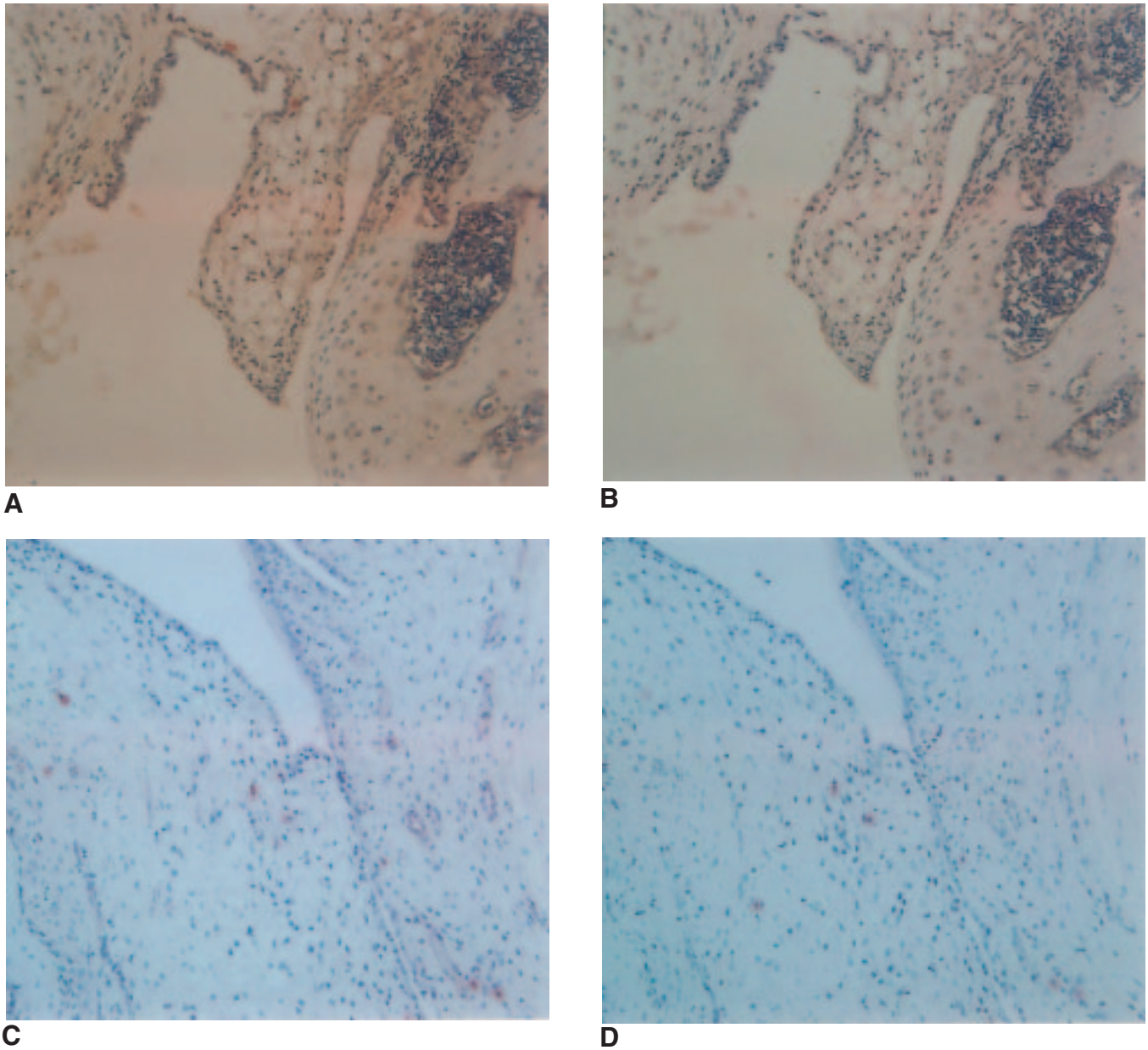


Figure 6. Immunohistochemical localization of MCP-1 and CCR2. Representative samples are shown of knee joint synovial tissue from a normal rat (A and B) and an AIA rat with erosive ankle arthritis (C and D). Sections were stained with AEC in PBS and counterstained with hematoxylin. There is immunoreactivity for MCP-1 (red color) in the normal joint cartilage and synovium (A). The diseased synovium shows positive immunoreactivity for MCP-1 (C) and CCR2 (D) (all original magnifications ×200).

adaptation for use in the specific joint space was difficult; and our technique has its limitations. The first was the calibration and assessment of relative recovery rates *in vitro* and *in vivo* in cytokine dialysis. Although the dialysis membrane had a characteristic 50 kDa molecular weight cutoff, the *in vitro* microdialysis recovery of IL-1ß was low. This low recovery might have caused underestimation of the level of cytokine expression, or even caused an artifact especially in *in vivo* microdialysis.

The small volume of microdialysate was the second limitation in our experiment. The ELISA system needed sufficient sample volume to determine the concentration of cytokine, so that it required more time for sample collection. Third, the rats were anesthetized during the entire microdialysis period, which is not optimal condition for a continuously performed study. Further, because of the invasive design of this procedure, we had to exclude the possibility that cytokine secretion was induced by the procedure itself. Fourth, although insertion of the probe resulted in minimal local trauma, the resulting mild inflammation and contamination from fluids such as blood might have affected the dialysate. However, technical limitations such as subtle vari-

ations in dialysis tubes and low recovery volumes could also have affected our results. Because of the dialysis limit, it would seem that small-size chemokines ought to dialyze into the dialysate more easily. We should therefore have detected more chemokines such as IL-8 or macrophage inflammatory protein, if they were present.

In inflammatory synovium, a complex network of cytokines regulates the pathogenesis of arthritis at different stages of disease progression. Although we may not observe these subtle changes clinically, cytokine expression is probably regulated during the course of the disease. It is also not clear why rats with AIA manifested bone erosions at the ankle but not at the knee, considering that both human disease (RA) and AIA in this model are systemic autoimmune diseases. Our results suggest that the knee joint may represent inflammation at the very beginning stage.

In our results, quantities of MCP-1 and IL-6 were detected in the microdialysates of both normal and arthritic rats; nevertheless these might be overestimated. A previous study had indicated that IL-6 and its soluble receptor might orchestrate the switch from an acute neutrophilic infiltrate to a chronic mononuclear cell population via MCP-1 upregulation during peritoneal inflammation[10], and this was consistent with arthritic joints[11]. We identified that MCP-1 was expressed at higher concentrations in rat knee joints at the late stages of AIA. Our results may corroborate those obtained by another group, which reported increased MCP-1 levels in ankle homogenates of AIA rats[6]. The expression of MCP-1 and its receptor was upregulated, which may indicate that many leukocytes were chemotactic to knee joint and enter an inflammation. The inability to detect IL-1ß and IFN-γ may indicate the real profiles of these cytokines in the late phase of AIA[6].

Investigators have reported methods to detect small molecules. For example, Miller, *et al*[1] used an *in vitro* superfusion method to detect IL-6 secretion in human synovial membrane. Lawand, *et al*[3] used an *in vivo* microdialysis method to detect excitatory amino acids in knee synovial perfusate by means of a Carnegie Medicine CMA/20 probe; Sjogren, *et al*[4] used an *in vivo* technique to determine IL-6 in human dermis. Those reports described cytokine dynamics and provided useful methods to study pathogenesis. *In vivo* microdialysis techniques have also been used in some diseases or animal models including amyotrophic lateral sclerosis[12], moya moya disease[13], McArdle's disease[14], Huntington's disease[15], Parkinson's disease[16], Alzheimer disease[17], and ischemic neuronal damage[18]. These reports suggest the *in vivo* microdialysis technique has extensive applications and therefore we would adapt this technique for use in research in RA.

Although AIA in rats is a well established model to study inflammatory arthritis, it is difficult to study the dynamic biochemical change taking place in synovial fluid. We know that many chemokines/cytokines are involved in the pathogenesis of RA, yet it is difficult to observe point changes in the inflammatory cascade. The *in vivo* microdialysis technique provides a way to carry forward those experiments investigating inflammation, which may lead to timely treatment with antiinflammatory cytokines.

Using *in vivo* microdialysis, we observed that MCP-1 exists in non-erosive knee joints of AIA rats, which had erosive changes in the ankle joints. Our findings suggest that MCP-1 might play a role in the early stage of progressive arthritis, and that the *in vivo* microdialysis technique could be adapted in research RA. At present, therapy for bone erosion in RA is still challenging[19,20] because there is no specific target to treat. Although we have no data to correlate disease severity with MCP-1, our results suggest that the animal model of knee joint erosion might be used to investigate the role of MCP-1 using *in vivo* microdialysis and that MCP-1 might be a target molecule for treatment of RA.

REFERENCES

1. Miller LE, Wessinghage D, Muller-Ladner U, et al. In vitro superfusion method to study nerve-immune cell interactions in human synovial membrane in long-standing rheumatoid arthritis or osteoarthritis. Ann NY Acad Sci 1999;876:266-75.
2. Humpel C, Ebendal T, Olson L. Microdialysis: a way to study in vivo release of neurotrophic bioactivity: a critical summary. J Mol Med 1996;74:523-6.
3. Lawand NB, McNearney T, Westlund KN. Amino acid release into the knee joint: key role in nociception and inflammation. Pain 2000;86:69-74.
4. Sjogren F, Svensson C, Anderson C. Technical prerequisites for in vivo microdialysis determination of interleukin-6 in human dermis. Br J Dermatol 2002;146:375-82.
5. Dayer JM, Bresnihan B. Targeting interleukin-1 in the treatment of rheumatoid arthritis. Arthritis Rheum 2002;46:574-8.
6. Szekanecz Z, Halloran MM, Volin MV, et al. Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. Arthritis Rheum 2000;43:1266-77.
7. Tylaska LA, Boring L, Weng W, et al. CCR2 regulates the level of MCP-1/CCL2 in vitro and at inflammatory sites and controls T cell activation in response to alloantigen. Cytokine 2002;18:184-90.
8. Luster AD. Chemokines — chemotactic cytokines that mediate inflammation. N Engl J Med 1998;338:436-45.
9. Van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum 2001;44:3-12.
10. Hurst SM, Wilkinson TS, McLoughlin RM, et al. IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. Immunity 2001;14:705-14.
11. Desgeorges A, Gabay C, Silacci P, et al. Concentrations and origins of soluble interleukin 6 receptor-alpha in serum and synovial fluid. J Rheumatol 1997;24:1510-6.
12. Bogdanov MB, Ramos LE, Xu Z, Beal MF. Elevated "hydroxyl radical" generation in vivo in an animal model of amyotrophic lateral sclerosis. J Neurochem 1998;71:1321-4.
13. Mendelowitsch A, Sekhar LN, Clemente R, Shuaib A. EC-IC bypass improves chronic ischemia in a patient with moyamoya disease secondary to sickle cell disease: an in vivo microdialysis study. Neurol Res 1997;19:66-70.
14. Vissing J, MacLean DA, Vissing SF, Sander M, Saltin B, Haller

RG. The exercise metaboreflex is maintained in the absence of muscle acidosis: insights from muscle microdialysis in humans with McArdle's disease. J Physiol 2001;537:641-9.
15. Bogdanov MB, Ferrante RJ, Kuemmerle S, Klivenyi P, Beal MF. Increased vulnerability to 3-nitropropionic acid in an animal model of Huntington's disease. J Neurochem 1998;71:2642-4.
16. Ferger B, Teismann P, Mierau J. The dopamine agonist pramipexole scavenges hydroxyl free radicals induced by striatal application of 6-hydroxydopamine in rats: an in vivo microdialysis study. Brain Res 2000;883:216-23.
17. Cirrito JR, May PC, O'Dell MA, et al. In vivo assessment of brain interstitial fluid with microdialysis reveals plaque-associated changes in amyloid-beta metabolism and half-life. J Neurosci 2003;23:8844-53.
18. Mendelowitsch A, Sekhar LN, Wright DC, et al. An increase in extracellular glutamate is a sensitive method of detecting ischaemic neuronal damage during cranial base and cerebrovascular surgery. An in vivo microdialysis study. Acta Neurochir (Wien) 1998;140:349-56.
19. Sokka T, Hannonen P. Healing of erosions in rheumatoid arthritis. Ann Rheum Dis 2000;59:647-9.
20. Jorgensen C, Noel D, Apparailly F, Sany J. Stem cells for repair of cartilage and bone: the next challenge in osteoarthritis and rheumatoid arthritis. Ann Rheum Dis 2001;60:305-9.