

# An Antagonist of Monocyte Chemoattractant Protein 1 (MCP-1) Inhibits Arthritis in the MRL-*lpr* Mouse Model

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## Summary

An antagonist of human monocyte chemoattractant protein (MCP)-1, which consists of MCP-1(9-76), had previously been characterized and shown to inhibit MCP-1 activity in vitro. To test the hypothesis that, by inhibiting endogenous MCP-1, the antagonist has antiinflammatory activity in vivo, we examined its effect in the MRL-*lpr* mouse model of arthritis. This strain spontaneously develops a chronic inflammatory arthritis that is similar to human rheumatoid arthritis. Daily injection of the antagonist, MCP-1(9-76), prevented the onset of arthritis as monitored by measuring joint swelling and by histopathological evaluation of the joints. In contrast, controls treated with native MCP-1 had enhanced arthritis symptoms, indicating that the inhibitory effect is specific to the antagonist. In experiments where the antagonist was given only after the disease had already developed, there was a marked reduction in symptoms and histopathology, although individuals varied in the magnitude of the response. The mechanism of inhibition of disease is not known, although the results suggest that it could be more complex than the competitive inhibition of ligand binding that is observed in vitro. The demonstration of the beneficial effects of an MCP-1 antagonist in arthritis suggests that chemokine receptor antagonists could have therapeutic application in inflammatory diseases.

Monocyte chemoattractant protein (MCP)<sup>1</sup>-1 is a chemoattractant cytokine (chemokine) (1) that promotes the migration and activation of monocytes (2, 3). It has been associated with several inflammatory diseases (4), but a causal relationship has been difficult to prove. Monocyte infiltrates are prominent in rheumatoid arthritis (RA) and their products, such as cytokines that amplify the inflammatory response and enzymes that destroy connective tissue (5), are readily detected in diseased joints. MCP-1 is produced by both synovial cells and infiltrated monocytes in RA (6–8). Thus, the inhibition of MCP-1 function could control inflammation by preventing monocyte accumulation in the joints.

To test the antiinflammatory effect of the MCP-1 antagonist, MCP-1(9-76) (9, 10), we chose a mouse model for RA. Previous studies had shown that murine monocytes respond to both human and mouse MCP-1 (11). The MRL-*lpr* mouse strain was chosen to test the antagonist because it has a genetic predisposition to arthritis with similar characteristics to human RA including cell infiltration, pannus formation, bone and cartilage breakdown, and the

presence of serum RF (12). The disease normally develops towards the end of the animal's life span (13); however, injection with CFA initiates early onset and increases the severity of arthritis, making the MRL-*lpr* mouse a practical experimental model for testing potential therapeutics (12).

## Materials and Methods

**The Proteins.** MCP-1, the antagonist, MCP-1(9-76), and the control peptide, MCP-1Ala, were chemically synthesized and characterized as described (9, 10). MCP-1Ala is an analogue of MCP-1 that had all the cysteines (residue numbers 11, 12, 36, and 52) replaced by alanines.

**Arthritis Induction and Treatment.** Both male and female MRL-*lpr* mice were used at 13–14 wk of age and were bred at the University of British Columbia (Vancouver, Canada) from stock originally obtained from the Jackson Labs. (Bar Harbor, ME). On Day 0 of each experiment, all groups of mice were injected with CFA intradermally into a thoracic and an inguinal site with 0.05 ml CFA supplemented to 10 mg/ml with heat inactivated *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI) (12). Either immediately or after a delay, depending on the experiment, mice were injected either intravenously, intraperitoneally, daily or not at all, with the appropriate antagonist or control protein. The chemokine analogue treatment was continued for 30 d. The ankle width was determined with a micrometer. For evaluation of the incidence of arthritis,

<sup>1</sup>Abbreviations used in this paper: CCR, human CC chemokine receptor; MCP, monocyte chemoattractant protein; RA, rheumatoid arthritis.

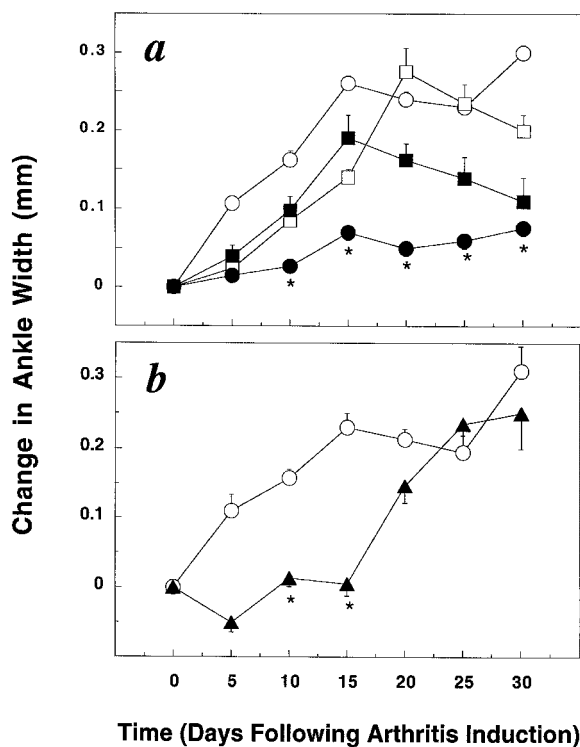
the symptoms of impaired mobility, presence of erythema, or swelling were scored as either + or -. Statistical analysis of the incidence was carried out with the one-tailed Fisher Exact test. For quantifying swelling, ankle widths were measured with a micrometer. The statistical comparison of paired sets of ankle width measurements was carried out using the Student's *t* test.

**Histopathological Analysis.** At day 30 after CFA priming, the hind paws were fixed in buffered formalin. After decalcification in 10% formic acid for 48 h, the tissues were processed for paraffin embedding. Serial sections of the tarso-metatarsal joints were cut to a thickness of 5  $\mu$ m and stained with hematoxylin and eosin. Sections were examined by an individual without knowledge of the experimental protocol. A minimum of 10 sections/joint were assessed and scored to provide a semiquantitative measure of sub-synovial inflammation (0, normal; 1, focal inflammatory infiltrates; 2, inflammatory infiltrate that dominated the cellular histology), synovial hyperplasia (0, normal; 1, a continuous, minimum three-layer thick, synovial lining seen in one joint; 2, minimum three-layer thick, synovial lining detected in several joints), pannus formation and cartilage erosion (0, normal; 1, pannus partially covered cartilage surfaces without evident cartilage loss; 2, pannus connected to evident cartilage loss), bone destruction (0, normal; 1, detectable destruction of bone by the pannus or osteoclast activity; 2, the pannus or osteoclast activity had destroyed a significant part of the bone), and finally, overall pathology was the overall assessment derived by the summation of the values for these criteria. Statistical analysis of the histopathology indices was done using the Student's *t* test.

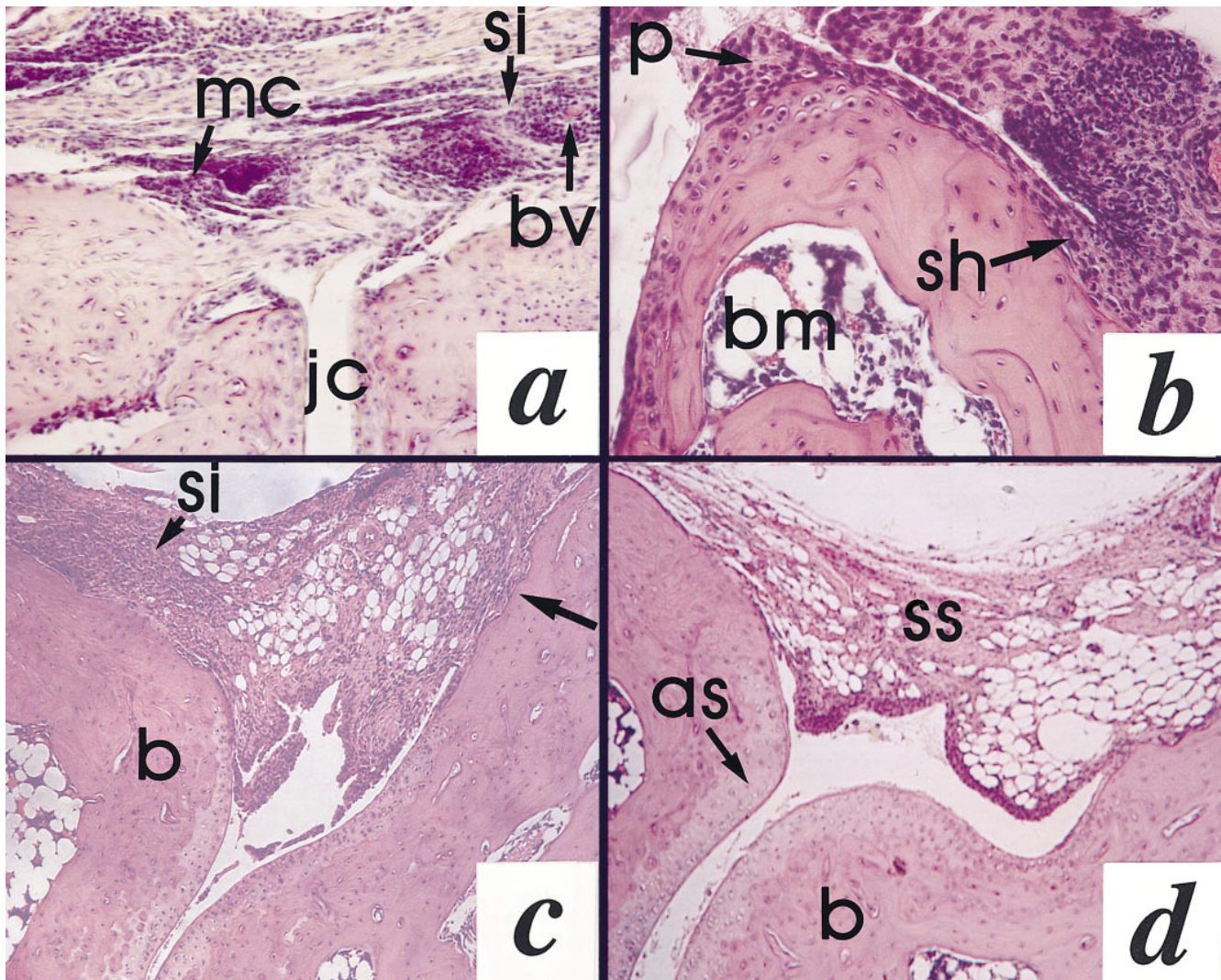
## Results

**Receptor Interactions of the MCP-1 Antagonist.** The human MCP-1(9-76) antagonist competed for binding of labeled MCP-1 to receptors on human monocytic cells with a dissociation constant ( $K_d$ ) of 8.3 nM (9). In vitro it inhibited MCP-1 (10 nM) with an  $ID_{50}$  of 70 nM (10). When tested for competition binding of a number of chemokines, the specificity of the antagonist was similar to that of native MCP-1. It is likely that the inhibitory effects of the MCP-1 antagonist are due to its binding to the human CC chemokine receptor (CCR)2 (14). The effects on mouse cells are probably due in part to blocking of the corresponding receptor, murine CC chemokine receptor 2, which appears to be similar to the human CCR2 in its specificity (15). Using a murine myelomonocytic cell line, WEHI 274, the  $K_d$ s of human MCP-1 and the MCP-1 antagonist were 39 nM and 58 nM, respectively. For the in vivo experiments, we injected MCP-1(9-76) so that theoretically there was a 13-fold (0.5 mg/kg) or a 54-fold (2.0 mg/kg) excess over the  $K_d$  measured on mouse cells, estimated on the basis of an average exchangeable fluid volume of 2 ml per mouse. MCP-1 was at a 72-fold excess. The control MCP-1Ala analogue had the same sequence as the antagonist except that all four cysteines were replaced by alanines. This analogue lacks the two essential disulfide bridges (Clark-Lewis, I., and J.-H. Gong, unpublished data), did not bind to MCP-1 receptor, and neither induced detectable chemotaxis, nor inhibited MCP-1 chemotaxis (data not shown). Thus the control, MCP-1Ala, was neither an agonist nor an antagonist.

**MCP-1 Antagonist Inhibits the Onset of Arthritis Symptoms.** To test the effect of the antagonist on the onset of disease, mice were primed with intradermal CFA on day 0. When the MCP-1 antagonist was given intraperitoneally at a dose of 2.0 mg/kg daily, it resulted in significant reduction of swelling of the ankle joint (Fig. 1 *a*). Controls that received the same dose of a closely related but inactive analogue, MCP-1Ala, developed similar swelling to that of untreated controls, but showed a trend towards a delayed onset. Although not significant, the effect was likely to be due to the daily injection protocol. In subsequent experiments, the controls were all injected with the control peptide, MCP-1Ala. In a separate experiment where the antagonist treatment was stopped at 15 d (Fig. 1 *b*), onset was inhibited during the treatment interval, but swelling became apparent by 20 d and rose to untreated control levels by 24 d. Mice that received a fourfold lower intraperitoneal dose of the antagonist did not have a significant reduction in swelling, suggesting that for maximal effect, the concentration of antagonist must be maintained at pharmacological levels. Analysis of the sera for anti MCP-1 antibodies



**Figure 1.** The MCP-1 antagonist prevents the onset of the symptoms of arthritis. The ankle widths for both hind legs of each animal were measured with a micrometer on the indicated days, and the results are presented as the mean change from the day 0 measurement,  $\pm$  SEM. \*Values are significantly different from the control MCP-1Ala analogue ( $P < 0.05$ ). (a) MRL-*lpr* mice received CFA on day 0 and were injected intraperitoneally daily for 30 d with either MCP-1 antagonist, 2.0 mg/kg,  $n = 23$ , ●; MCP-1 antagonist, 0.5 mg/kg,  $n = 9$ , ■; control MCP-1Ala analogue, 2.0 mg/kg,  $n = 10$ , □; or no further treatment,  $n = 20$ , ○. (b) Mice were either injected intravenously daily for 15 d with MCP-1 antagonist, 0.5 mg/kg,  $n = 6$ , ▲; or received no further treatment,  $n = 8$ , ○. Results shown are from one of two similar experiments.



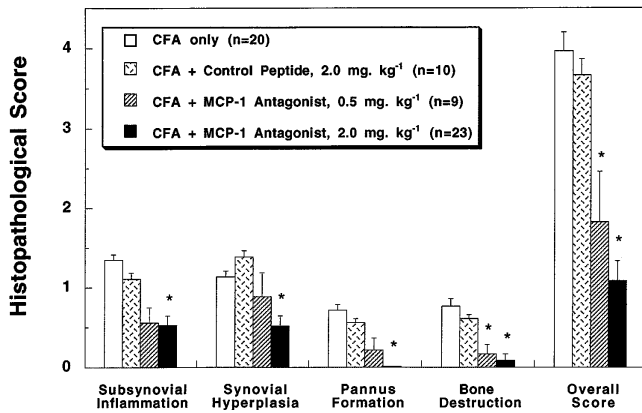
**Figure 2.** The effects of MCP-1 antagonist on the joint histopathology. Representative photomicrographs of histology from the experiment in Fig. 1 *a* are shown. For the control group: (*a*) marked subsynovial infiltration by mononuclear cells resembling rheumatoid nodule formation ( $\times 20$  objective); (*b*) pannus formation and synovial hyperplasia ( $\times 40$  objective); (*c*) bone erosion (*arrow*;  $\times 20$  objective). For the antagonist (2.0 mg/kg) -treated group: (*d*) indicates some stromal thickening of the subsynovium, but absence of infiltrating cells or joint destruction ( $\times 20$  objective). *as*, articular surface; *b*, bone; *bm*, bone marrow; *bv*, blood vessel; *jc*, joint cavity; *mc*, mononuclear cell infiltrate; *p*, pannus; *sh*, synovial hyperplasia; *si*, subsynovial inflammation; *ss*, subsynovium.

showed that low titers (1:160) were present in both groups (not shown). The production of anti-MCP-1 antibodies was not considered a major factor in these studies.

**Effects of the Antagonist on Joint Histopathology.** Histological analysis of the ankle joints was performed at day 30 for all the experiments described. Shown in Fig. 2 are photomicrographs taken from representative antagonist-treated and control animals, from the experiment described in Fig. 1 *a*. The effects observed in control mice that were not given MCP-1 antagonist included infiltration of mononuclear cells into the subsynovial tissue (Fig. 2 *a*), synovial hyperplasia, pannus formation (Fig. 2 *b*), and bone erosion (Fig. 2 *c*). In contrast, mononuclear cell infiltration and bone and cartilage pathology was absent in the MCP-1 antagonist-treated example. Only minor thickening of the subsynovium can be seen (Fig. 2 *d*). Analysis of the histo-

logical results (Fig. 3) for the experiment described in Figure 1 *a*, indicates that the group that received 2.0 mg/kg antagonist had significant lower subsynovial inflammation, synovial hyperplasia, pannus formation and cartilage erosion, bone destruction, and overall histopathology. The histopathology results for the antagonist-treated group compared favorably to age-matched animals that have not been primed with CFA and have no disease symptoms (not shown). Interestingly, compared to the controls, the group (Fig. 1 *a*) that received 0.5 mg/kg had significantly lower overall histopathology (Fig. 3), even though joint swelling was not significantly reduced. This suggests that the antagonist is affecting cellular infiltration and pathology even in the absence of an apparent effect on externally measurable symptoms.

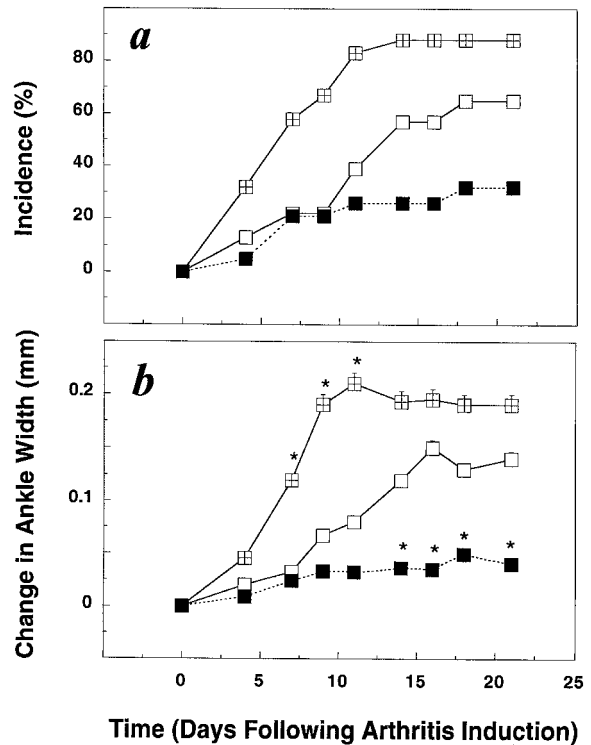
**Native MCP-1 Enhances Arthritis.** To more completely



**Figure 3.** Summary of the effects of MCP-1 antagonist on joint histopathology. Shown are the mean  $\pm$  the SEM of the scores, from the experiment in Fig. 1 a, determined as described in Materials and Methods. \*Groups that were significantly different ( $P < 0.05$ ) from the control MCP-1Aa analogue for each histopathological parameter.

describe this effect and to provide some insight into possible mechanisms, we examined the effect of full-length functional MCP-1 in this model. Native MCP-1 treatment significantly enhanced the disease, as indicated by the earlier and higher incidence of disease (Fig. 4 a) and magnitude of swelling (Fig. 4 b), compared to the inactive control protein. Thus, MCP-1 is causing hyperresponsiveness to arthritis in the MRL-*lpr* mouse, and is having the opposite effect to the antagonist when both are compared to the inactive control peptide. In contrast to the experiments described in Figs. 3 and 6, for this experiment, histopathological analysis was done 7 d after onset of symptoms and the time varied from animal to animal. At this time point there was no significant difference in the histopathology between the MCP-1 and control groups, although the trend was toward more histopathology in the MCP-1 group, which is consistent with the observed enhancement of swelling (not shown). The MCP-1 antagonist significantly reduced inflammatory infiltration, hyperplasia, bone destruction, and overall histopathology in this experiment.

**MCP-1 Antagonist Inhibits Symptoms of Disease when Given after Onset.** Although the experiments described so far demonstrate that the MCP-1 antagonist is capable of preventing the onset of arthritis, they do not show whether the antagonist inhibits disease that is already evident. The postonset situation is a much more stringent test of the ability of the antagonist to inhibit disease, as cell infiltration and inflammatory events are already going on when the antagonist treatment is started (12). Another reason to address the postonset effects is that this more closely reflects the clinical situation where symptoms are already apparent when a patient presents with RA. To test the effects of the antagonist on existing disease, we primed the mice at day 0, but then delayed treatment until after significant swelling was apparent. The results for representative individuals from the control peptide- (Fig. 5 a), and antagonist- (Fig. 5 b) treated groups are shown. The individuals from the control group generally had recurrent swelling after symptoms first be-



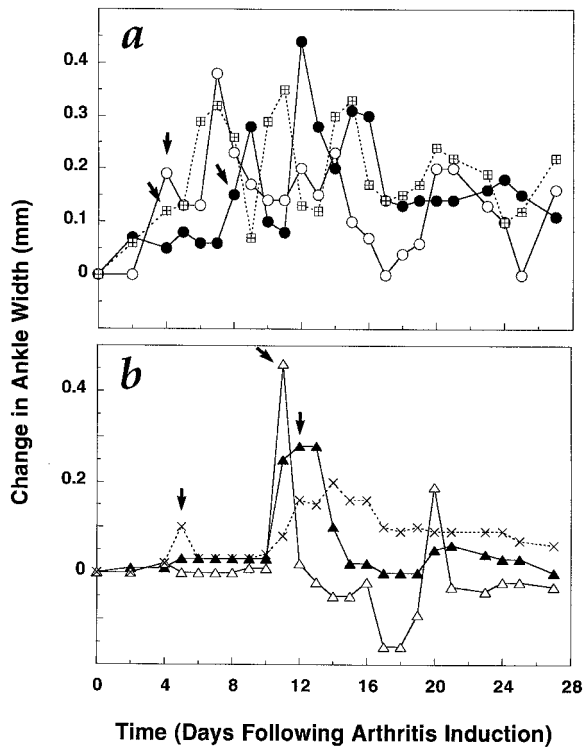
**Figure 4.** The effects of the functional MCP-1 agonist on disease incidence and joint swelling. The mice were given CFA treatment at day 0 and divided into three groups. The groups received daily injection of 2 mg/kg of either native MCP-1 (1-76),  $n = 24$ , □; MCP-1 antagonist,  $n = 19$ , ■; or the inactive control peptide,  $n = 23$ , □. (a) The incidence as percentage of mice that showed visible erythema or swelling on the days indicated. (b) The change in the ankle widths (mean  $\pm$  SEM). \*Significantly different from controls ( $P < 0.05$ ).

came apparent. This is typical of the disease in this model. Individuals from the treated group showed an immediate reduction of swelling and duration of inflammatory episodes. Some degree of relapse of the antagonist-treated animals was apparent at later time points, and the duration and persistence of this was highly variable. Nevertheless, when all the animals were taken into account (not shown), the results indicated that the antagonist greatly reduced the symptoms.

MCP-1 antagonist treatment significantly reduced joint histopathology by day 28. Thus, synovial hyperplasia, subsynovial inflammation, cartilage erosion, and overall histopathology were significantly reduced in the antagonist treated group (Fig. 6). This indicates that the external observation of reduction of swelling in mice treated after disease onset is reflected in the inflammatory disease in the joints, as measured by histopathology.

## Discussion

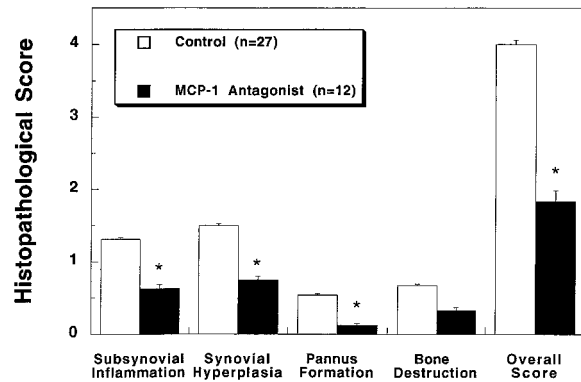
In this study, we have shown that a human MCP-1 receptor antagonist greatly reduces the symptoms and histopathology of chronic arthritis in a disease model. Infiltration of the subsynovium by monocytes is prominent in



**Figure 5.** The effect of treatment with MCP-1 antagonist after disease onset. Animals were injected with CFA once at day 0 and examined daily for the visual appearance of erythema and swelling around the joints as measured with a micrometer. 23 of 40 mice developed swelling at different times, and they were injected daily with 2.0 mg/kg of either the MCP-1 antagonist or the MCP-1Ala control, on the day indicated by the arrows, until day 28. (a) The swelling measurements of three representative examples from the 13 controls (●, ○, ⊞). (b) Three representative examples from the 10 antagonist-treated mice (▲, △, X).

human RA, and thus, the diminished cellular infiltration observed in these studies is probably due to inhibition of endogenous MCP-1 receptors (see below). The results indicate that the effect of the antagonist is reversible, because when the antagonist treatment was stopped, the swelling symptoms return. Moreover, antagonist treatment inhibited the disease after symptoms were already apparent, suggesting that there is turnover of cells that infiltrate the lesion and that inhibition of further infiltration leads to reversal of symptoms and pathology.

Native MCP-1 accelerated the onset and enhanced the symptoms of joint inflammation. Predictions could have been made, not only for the hyperresponsiveness that was observed, but also for inhibition similar to that seen with the antagonist. In vitro, MCP-1 results in migration only if a gradient is formed and if at high concentrations, MCP-1 inhibits migration by collapsing the gradient (16). Moreover, both the agonist and the antagonist desensitize MCP-1 receptor signaling in vitro (4, 9). Nonresponsiveness has also been reported in vivo for IL-8 (17). It is unlikely that the enhancement we have observed in vivo with MCP-1 is simply nonspecific, since the control protein, which is similar chemically, did not cause this effect. Rather, it is more likely that the systemic levels of MCP-1 in these mice are



**Figure 6.** The effect of postonset treatment with MCP-1 antagonist on joint histopathology. Histopathological assessments of the experiment outlined in Fig. 5 were made 28 d after CFA priming. All the mice that developed significant swelling are included. The data is presented as for Fig. 3. Shown are the mean  $\pm$  SEM of the assessment values for the indicated parameters. \*The antagonist and the controls are significantly different,  $P < 0.05$ .

insufficient to cause nonresponsiveness of the target cells to MCP-1. Our interpretation for the enhancement of the onset and swelling is that the injected MCP-1 accumulates in the tissues and causes activation of monocytes and other target cells, whereas the MCP-1 that remains in the vascular compartment is eliminated rapidly. A gradient of MCP-1 from the abluminal side (high) to the luminal side of the endothelium (low) could result in migration of responsive cells from the blood into the tissues. Monocyte activation could lead to some of the pathological effects observed with MCP-1.

The results suggest that the in vivo action of the antagonist is dependent on its receptor binding and its inability to cause activation. However, it cannot be directly proven that the receptor blocking competition that occurs in vitro, is also the mechanism in vivo. A case could be made for a more complex mechanism. Physiologically, the CC chemokine receptor system is redundant in that multiple CCRs, which each bind several ligands, are coexpressed (18). Thus, other chemokines and their receptors can induce similar activities in vitro. Since only CCR2 receptor is inhibited by the MCP-1 antagonist, then the question arises: why are we not seeing migration in response to other chemokines, such as RANTES, that bind to CCR1, CCR3, CCR4, and CCR5, but not CCR2 (18)? RANTES is also produced in inflamed joints along with the MCP-1 (19). The answer is not known. However we have found that a RANTES antagonist, RANTES (9-68), also inhibits the onset of arthritis in the model described here and, to a similar extent, as the MCP-1 antagonist. The fact that two antagonists that bind different receptors have similar effects suggests that the independence of the receptor actions that is observed in vitro is not directly translated to the in vivo situation. It is possible that the long-term presence of antagonist not only prevents cells from responding to chemokine, but also prevents migration by another mechanism. The antagonists do not stimulate detectable receptor signaling, but likely promote receptor internalization, and there

may be separate negative regulatory effects caused by receptor occupancy. Some of the possible mechanisms for nonresponsiveness of chemokines have been reviewed (4). Some that could apply to the MCP-1 antagonist are heterologous desensitization of receptors, failure to stimulate adhesion molecules (20), changes to the cytoskeleton (21), or general interference of the formation of endogenous chemokine gradients due to saturation of glycosaminoglycan interaction sites (22). Another possibility is that chemokine production is turned off by the presence of excess antagonist. This could occur if MCP-1-responsive cells have not migrated, and therefore cannot amplify chemokine and cytokine production. Further work will be necessary to determine the in vivo mechanisms of chemokine antagonist action. Nevertheless, our results indicate that the in vitro patterns of receptor specificity and chemokine function do not always correspond to in vivo effects.

Infiltrated monocytes are thought to be important in the pathology of RA (5). Although MCP-1 primarily acts on monocytes, it is also known to stimulate basophils (23) and T lymphocytes (24), indicating that these cells could also be stimulated by MCP-1 and/or be inhibited by the MCP-1 antagonist. Whatever target cells are important in this arthritis model, the results suggest that blocking MCP-1 receptors breaks a critical link in the chain of inflammatory events. Furthermore the antagonist prevents arthritis onset and also alleviates existing disease, suggesting the potential for MCP-1 antagonists or other cytokine inhibitors in the therapy of the human disease. Antibody therapy represents

an alternative approach to inhibition of ligand function (25). Beneficial effects in RA of antibodies that block tumor necrosis factor- $\alpha$  activity have been described (26). However, a potential disadvantage of the antibody approach in typical pathological situations is that targeting just one ligand may not be effective. On the other hand, with the antagonist approach, all the ligands for the receptor are blocked. For example, the MCP-1 antagonist described here blocks not only MCP-1 activity (9), but also MCP-2 (Gong, J.-H. and I. Clark-Lewis, unpublished observations) and MCP-3 (10).

Receptor antagonists that inhibit ligand binding and function is a conventional pharmacological approach that relies on competition between the antagonist and the natural agonist(s) for specific receptor binding sites. Antagonists and agonists for seven transmembrane receptors, a class that includes all the chemokine receptors, form the basis for many widely used pharmaceuticals (27). Targeted receptors include, for example, those for histamine, epinephrine, and serotonin. Most of these antagonists are nonpeptide in nature and are orally active, a major advantage for therapeutic use. Nevertheless, nonpeptide antagonists of peptide ligands, including neurokinins, cholecystokinin, and angiotensin, have also been developed (27). Despite the fact that chemokines are larger than these peptides, our results have indicated that the major binding site on MCP-1 and other chemokines is relatively small (9, 28), suggesting that nonpeptide antagonists for chemokine receptors may be a future possibility.

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