Effects of Interleukin-1 Receptor Antagonist in a Slow-Release Hylan Vehicle on Rat Type II Collagen Arthritis

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Purpose. To determine the effect of hylan fluid (HA), a model slow release vehicle on the pharmacokinetic profile and efficacy of interleukin-1 receptor antagonist (IL-1ra) in rats with established type II collagen arthritis.

Methods. Female Lewis rats with type II collagen arthritis were treated daily, every other day or every third day with single subcutaneous (sc) injections of IL-1ra formulated in HA and the effects on arthritis determined. Results were compared to those obtained with IL-1ra in citrate buffered saline with EDTA and polysorbate (CSEP). Sequential blood levels were determined in rats injected sc with IL-1ra in CSEP or HA.

Results. Incorporation into HA led to slower release of IL-1ra into the bloodstream and maintained therapeutic blood levels of IL-1ra for a longer time compared to the IL-1ra/CSEP formulation. Single daily sc doses of 100 mg/kg IL-1ra in CSEP were ineffective in type II collagen arthritis. By contrast, once per day dosing of 100 mg/kg IL-1ra in HA provided 78% inhibition of paw swelling. Every other day dosing with 100 mg/kg IL-1ra in HA resulted in 62% inhibition. IL-1ra (100 mg/kg in HA) given every third day provided 19% inhibition of arthritis. Improved efficacy correlated with improved pharmacokinetics.

Conclusions. Administration of IL-1ra in the slow release vehicle HA improves pharmacokinetics and efficacy in rat type II collagen arthritis.

INTRODUCTION

Interleukin-1 receptor antagonist (IL-1ra) is a 17.2 kd, 152 amino acid protein that binds the IL-1 receptor and blocks the action of IL-1, a proinflammatory cytokine (1). A methionylated version of this protein (rhIL-1ra, 153 amino acids) currently in clinical trials for the treatment of rheumatoid arthritis (RA) has shown some beneficial effects as evidenced by decreased pain, number of swollen joints, and C-reactive protein levels in patients in a phase II study (2). The protein was administered by single daily sc injections.

Rat type II collagen arthritis, an animal model of RA, results when rats are immunized against homologous or heterologous type II collagen. The resulting polyarthritis is characterized by marked cartilage destruction associated with immune complex deposition on articular surfaces, bone resorption and periosteal proliferation, and moderate to marked synovitis and periarticular inflammation (3). The lesions in type II collagen arthritis are analogous to those seen in human RA (4) and the model has been used to predict the activity of various agents currently used in the treatment of RA.

IL-1ra administered by continuous sc infusion (1–5 mg/kg/hr) to provide plasma levels ranging from 1–5 µg/ml results in 50–90% inhibition of established rat collagen arthritis (Bendele et. al. unpublished data). IL-1ra in its liquid vehicle CSEP, has a short plasma half life after sc injection typical of freely filtered proteins of this molecular weight (5). Therefore, in animal arthritis studies, sc injections of fairly high doses of IL-1ra in CSEP generally have to be given at least twice a day to achieve some efficacy. In our clinical studies in which the protein is given by sc injection once a day, plasma levels peak at 6 hrs. and fall into the 10–500 ng/ml range within 24 hours. These levels are well below those required for optimal activity in animal models of arthritis. Since continuous IL-1 receptor blockade appears to be important in optimizing clinical efficacy in animal models, we hypothesized that administration of IL-1ra in a slow release vehicle might provide the sustained blood levels needed for optimal clinical efficacy.

Hyaluronic acid is a large linear glysoaminoglycan (GAG) composed of repeating units of glucuronic acid and N-acetylglucosamine. Depending on the method of preparation and the source, the molecular weight of hyaluronic acid can vary from 50,000 to over 4×10^6 and exhibits excellent biocompatiblity (6,7,8). Hyaluronic acid has useful rheological properties including shear-dependent viscosity which allows even very concentrated solutions to be pushed through small-bore needles (9). Several derivatives of hyaluronic acid have been prepared in order to improve the physical properties or prolong the in vivo degradation time. For example, hyaluronic acid esters and cross-linked hyaluronic acid derivatives, known as hylan (HA), have been prepared and used to deliver a variety of therapeutic agents including steroids (10), insulin (11), insulin-like growth factor (12), interferon (6) nerve growth factor (NGF) (13) and granulocyte colony stimulating factor (G-CSF) (14). In the present studies, we formulated IL-1ra in HA. We find that HA slowly releases IL-1ra into the bloodstream after sc injection and that IL-1ra/HA given daily or every other day was more efficacious in inhibiting disease progression in established rat type II collagen arthritis than the liquid IL-1ra formulation.

MATERIALS AND METHODS

Animals

Female Lewis rats (200–250 g, Charles River, Portage, MI) were used in these studies. Animals were allowed to acclimate for at least 3 days prior to initiation of experimentation. Rats were housed 4/cage in shoebox style cages and were allowed *ad libitum* access to food and water. All animal use was in accordance with USDA guidelines for humane care. The research adhered to the "Principals of Laboratory Animal Care" (NIH publication #85–23, revised 1985).

Materials

Recombinant IL-1ra and its vehicle were produced at Amgen, Boulder, CO. HA was purchased from Biomatrix, Inc.

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(Ridgefield, NJ) and formulated with IL-1ra at Amgen. In pre-liminary studies, IL-1ra (100 mg/ml) was formulated with 0.5, 1 and 2% HA. Initial testing (data not shown) of these various concentrations of HA in pharmacokinetic studies revealed that the 2% HA formulation consistently resulted in excellent sustained blood levels as compared to CSEP and the other formulations. Therefore, this formulation was evaluated for efficacy in the arthritis models. Freunds incomplete adjuvant (ICA) was obtained from Difco. Type II collagen was purchased from Elastin Products (Owensville, MO.) and endotoxin (LPS type L-3129) was from Sigma.

Induction of Collagen Arthritis

Female rats were given intradermal injections of bovine type II collagen (2 mg/ml in incomplete Freund's Adjuvant) at the base of the tail and over the back in 3 sites (250 μl divided) on day 0 and day 7. On day 12 they were given an IP injection of endotoxin (3 mg/kg). Onset of arthritis occurred over the next 5 days and as rats developed disease they were randomized to study groups (6–8/group) and treatment was initiated. Rats were treated for 6–7 days (subcutaneous injections in dorsum of the back) and then terminated on day 7 or 8 of arthritis for assessment of paw weights and tissue collection.

Clinical Assessment of Collagen Arthritis

In all studies, there was a group of normal nonarthritic rats (N = 4) that were treated with vehicle. These were used for paw diameter and final weight comparisons in calculation of % inhibition of arthritis. All studies had animals with arthritis that were treated with the appropriate vehicle control solution using the same dosing regime as was given to the IL-1ra treated rats (N = 7–8) and these animals were also used in the calculation of % inhibition. The remaining groups consisted of arthritic rats that received the various treatments under investigation. Caliper measurements of ankle joint width were done prior to onset of arthritis, on the day of randomization and on each subsequent study day until termination of the study on arthritis day 7. The data were then expressed as area under the curve (AUC) for purposes of determining % inhibition from controls over the duration of the arthritis. For calculation of AUC, the daily width of ankle joints for each rat was entered and plotted with the Statistical Analysis Software (SAS, Cary, NC) where the area between the treatment days after the onset of disease to the termination day was computed. Means for each group were determined and % inhibition from arthritis controls was calculated by comparing values for treated and normal animals. At termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for determination of final paw weights as another measure of inflammation. Ankle joints were then collected into formalin for histopathologic evaluation. % Inhibition of paw weight and AUC was calculated using the following formula:

$$\% \text{ Inhibition} = A - B/A \times 100$$

A = Group mean value for disease control − Group mean value for normal

B = Group mean value for treated − Group mean value for normal

Histopathology

Ankle joints were collected into 10% neutral buffered formalin for at least 24 hours prior to placement in Surgipath decalcifier I (Surgipath, Grayslake, IL.) for approximately 1 week. When decalcification was complete, the digits were trimmed and the ankle joint was transected in the longitudinal plane to give approximately equal halves. These were processed for paraffin embedding, sectioned and stained with hematoxylin and eosin for general evaluation of inflammation and bone damage and stained with toluidine blue for specific evaluation of cartilage changes according to criteria outlined in Table 1.

Plasma IL-1ra Determination

Blood samples for plasma level determination of IL-1ra were collected from jugular cannulas at various times from rats (N = 8/group) given single sc doses of 100 mg/kg IL-1ra in CSEP or HA (2%). Samples were analyzed using an ELISA method with an antibody to IL-1ra produced at R&D Systems. This assay employs the quantitative sandwhich enzyme immunoassay technique. Microtiter plates are coated with a monoclonal antibody specific for r-metHuIL-1ra and then blocked with 2% bovine serum albumin/phosphate buffered saline. Standards and samples are added to the wells and any r-metHuIL-1ra present is bound by the immobilized antibody. After washing away any unbound proteins, an enzyme linked polyclonal antibody specific for r-metHuIL-1ra is added to the wells to sandwich the r-metHuIL-1ra immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of r-metHuIL-1ra bound in the initial step. The color development is stopped and the intensity of color is measured. A curve is prepared, plotting the optical density versus the concentration of r-metHuIL-1ra in the standard wells. By comparing the optical density of the samples to this standard curve, the concentration of the r-metHuIL-1ra in the unknown samples is then determined. The sensitivity of this assay is <1.57 ng/ml when corrected for dilution.

Statistical Analysis

Clinical data for ankle width was analyzed by determining the area under the dosing curve with subsequent analysis of variance. Paw weights (mean ± SE) for each group were analyzed for differences using the Student's T Test. In both cases, significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Rats with established type II collagen arthritis given single daily sc doses of 100 mg/kg IL-1ra in CSEP had no significant inhibition of paw swelling over time (Fig. 1). Likewise, no significant decreases in paw weights were observed (Fig. 2). In other studies (Bendele, unpublished data) in which IL-1ra was administered by continuous sc infusion, the ED_{50} for suppression of clinical arthritis was 1 mg/kg/hr, a dose which maintains blood levels of approximately 1 μg/ml. Significant but lesser (32%) inhibition occurs when blood levels drop to

Table 1. Histopathology Scoring Criteria for Inflammation, Cartilage Damage and Bone Resorption

Score	Inflammation	Cartilage damage	Bone resorption
0	None present	None present	None present
1	Minimal infiltration in periarticular tissues	Minimal to mild loss of T. Blue staining no chondrocyte loss or collagen damage	Minimal small areas of resorption, rare osteoclasts
2	Mild infiltration in periarticular tissues	Mild loss of T. Blue staining with focal chondrocyte loss and collagen damage	More numerous areas of resorption in trabeculae and cortex, not full thickness
3	Moderate infiltration with moderate edema	Moderate loss of T. Blue staining with mid zone chondrocyte loss and collagen damage	Obvious resorption of medullary trabecular and cortical bone w/o full thickness defect in cortex, numerous osteoclasts
4	Marked infiltration with marked edema	Marked loss of T. Blue staining with deep zone chondrocyte loss and collagen damage	Similar to 3, but with full thickness defect in cortical bone
5	Severe infiltration with severe edema	Severe diffuse loss of T. Blue staining with multifocal severe (to tide mark) chondrocyte loss and collagen damage	Similar to 4, but with marked resorption in small tarsal bones

0.2 µg/ml. In order to achieve similar results with bolus sc dosing of IL-1ra in CSEP, animals must be injected every 8 hrs. Typically in most models and test systems, a high molar excess of IL-1ra must be present to insure near complete receptor blockade since only 2–3% of receptors need to be occupied in order for IL-1 to have biologic effects (15).

Results of our blood level analysis of rats given a single dose of IL-1ra in CSEP (Fig. 3) show that IL-1ra levels are below 0.1µg/ml 12 hrs. post dosing. When IL-1ra was given in the 2% HA vehicle, blood levels were greater than 1 µg/ml for the first 12 hours after dosing and were above 0.2 µg/ml for the last 12 hrs. of the dosing period (Fig. 3). In contrast to the lack of efficacy when single daily 100 mg/kg doses of IL-1ra in CSEP were given, administration of single daily sc doses of 100 mg/kg IL-1ra in HA resulted in 62% inhibition of paw swelling over time and 74% inhibition of final paw weights (Figs. 1, 2). These results clearly demonstrate the superior clinical effects of daily dosing of IL-1ra in HA vs. in CSEP. In addition, histologic analysis of ankle joint sections revealed
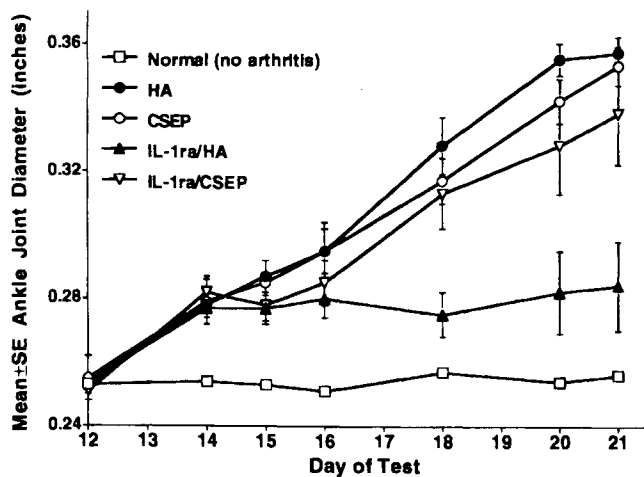

Fig. 2. Effects of once daily sc (QD) IL-1ra in HA (100 mg/kg) vs. IL-1ra in CSEP (100 mg/kg) shown in comparison to HA or CSEP vehicle alone on final paw weights in rats with established type II collagen arthritis. *p ≤ 0.05, 2 tailed T test, comparison to arthritis control, % on bars = % inhibition from arthritis control, N = 8/arthritis group, = 4/normal controls

Fig. 1. Effects of once daily (QD) IL-1ra in HA (100 mg/kg) vs. IL-1ra in CSEP (100 mg/kg) sc shown in comparison to HA or CSEP vehicle alone on ankle joint diameter over time in rats with established type II collagen arthritis (N = 8/group). Normal (nonarthritic) controls (N = 4) were given daily injections of HA.
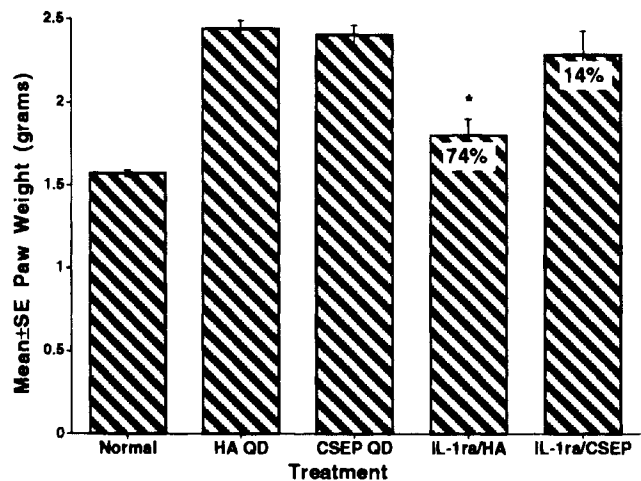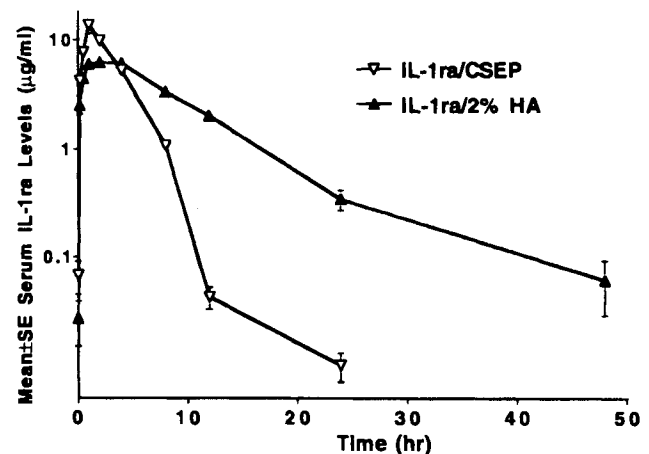

Fig. 3. Serum levels of IL-1ra in rats given 100 mg/kg IL-1ra in HA or CSEP sc and bled sequentially over time (N = 8/group).
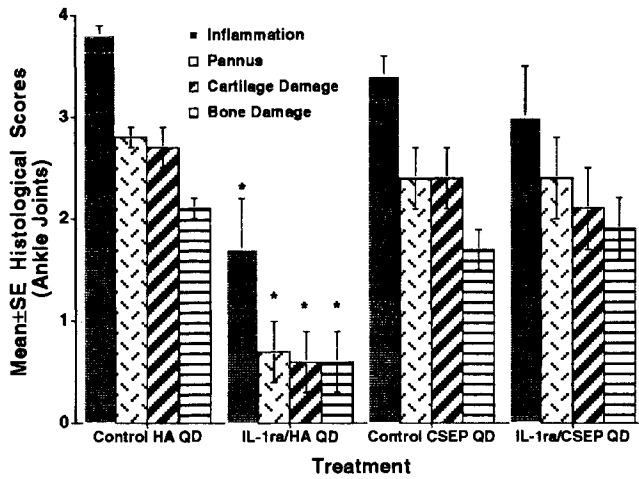
Fig. 4. Effects of IL-1ra in HA (100 mg/kg) vs. IL-1ra in CSEP (100 mg/kg) sc QD on histologic features of arthritis in rats with established type II collagen arthritis. *p ≤ 0.05, 2 tailed T test, comparison to arthritis control.

Fig. 6. Effects of sc once daily (QD), every other day (Q2D) or every third day (Q3D) IL-1ra in HA (100 mg/kg) shown in comparison to HA (QD) or no treatment on area under the curve from Fig. 5 (ankle joint diameter over time) in rats with established type II collagen arthritis. *p ≤ 0.05, ANOVA, comparison to arthritis control treated with HA, % on bars = % inhibition from arthritis control. N = 7/arthritis group, = 4/normal controls.

marked decreases in inflammation, pannus formation, and cartilage and bone damage in rats treated with IL-1ra in HA but not CSEP (Fig. 4).

After confirming that single daily sc doses of IL-1ra in HA were able to modulate disease progression, studies were done to determine the duration of effect. Rats treated with 100 mg/kg IL-1ra in HA every day had 53% inhibition of paw swelling over time and 78% inhibition of final paw weights (Figs. 5, 6, 7). Arthritic rats treated every other day with 100 mg/kg IL-1ra in HA had 35% inhibition of paw swelling over time and 62% inhibition of final paw weights. Arthritic rats treated with 100 mg/kg IL-1ra in HA every third day had 27% inhibition (nonsignificant) of swelling over time and 19% inhibition of paw weights. These results again demonstrate the importance of maintaining minimal blood levels of at least 200
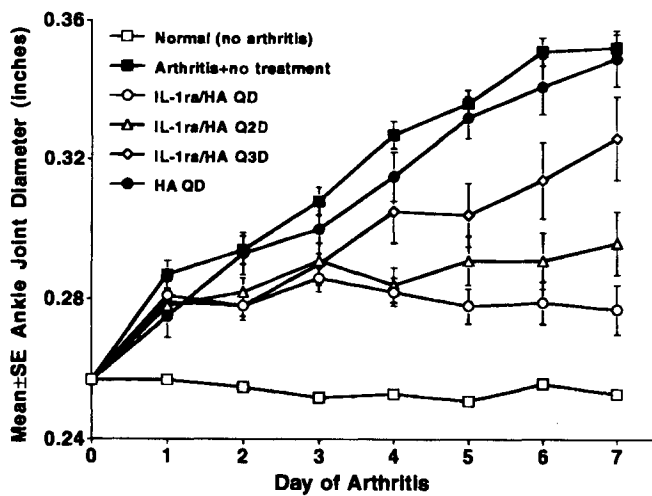
ng/ml during the period of time in which IL-1 is important in the pathogenesis in the model. Blocking the IL-1 receptor intermittantly results in less efficacy. Rats treated every third day, were dosed on day 1 and day 4 of arthritis. Interestingly, caliper measurements done 24 hrs. post dosing (day 2 and day 5) indicate suppression of arthritis progression (Fig. 5). However, measurements taken 2 or 3 days post dosing prior to rats being given their next dose, reflect disease progression, presumably as a result of the less than optimal blood levels during that period of time.

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of the joints with concomitant destruc-

Fig. 5. Effects of sc once daily (QD), every other day (Q2D) or every third day (Q3D) IL-1ra in HA (100 mg/kg) shown in comparison to HA (QD) or no treatment on ankle joint diameter over time in rats with established type II collagen arthritis (N = 7/group). Normal (nonarthritic) controls were given daily injections of HA (N = 4).
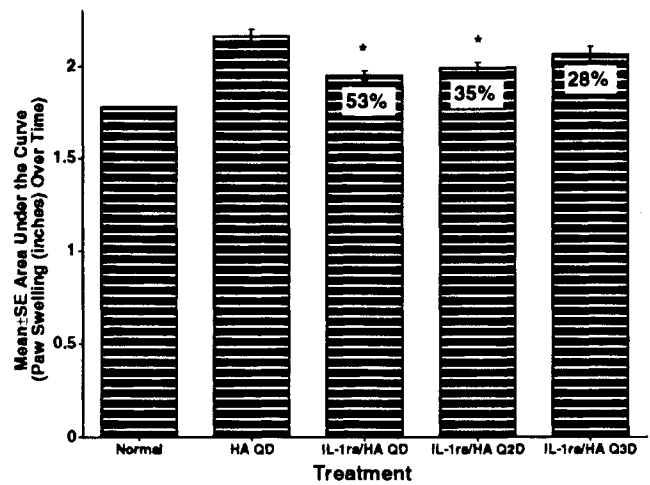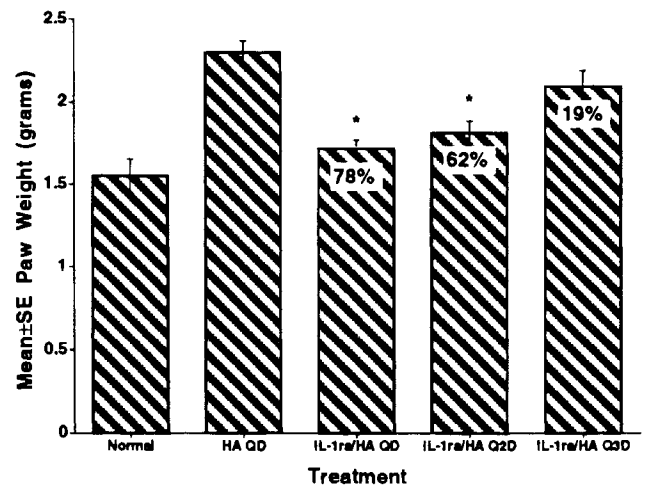

Fig. 7. Effects of sc once daily (QD), every other day (Q2D) or every third day (Q3D) IL-1ra in HA (100 mg/kg) shown in comparison to HA (QD) treatment on final paw weight in rats with established type II collagen arthritis. *p < 0.05, 2 tailed T test, comparison to arthritis control treated with HA, % on bars = % inhibition from arthritis control. N = 7/arthritis group, = 4/normal controls.

tion of cartilage and bone. The involvement of cytokines, particularly IL-1 and TNF, in the pathogenesis of RA is now well accepted as a result of numerous studies in animal models as well as in patients with the disease (16–19). The IL-1ra is a specific receptor antagonist that competitively inhibits binding of IL-1α and IL-1β to human and animal type I and II IL-1 receptors (1). Clinical trials have been completed in which IL-1ra has been administered chronically to patients with RA. Results indicate that treatment with IL-1ra moderately lowers acute phase proteins, swollen joint counts and may inhibit radiographic disease progression. These effects were achieved with daily sc dosing, (approximately 2 mg/kg) a regimen which results in peak plasma levels of approximately 1.6 μg/ml at 6 hrs. post dosing with levels less than 1 μg/ml after 18 hrs. (Bendele et. al., unpublished data). These blood levels are in the range of levels that would be predicted to give modest (30–50% inhibition) anti-inflammatory effects in rat type II collagen arthritis. The question then becomes whether efficacy in humans can be improved by prolonged maintenance of IL-1ra blood levels as was done in rat arthritis using continuous infusion or slow release vehicles. Our current results demonstrate that administration of IL-1ra in HA as a slow-release vehicle with maintenance of higher blood levels over time, definitely improves efficacy over that seen with single doses of IL-1ra in CSEP. These results also suggest that in addition to improving efficacy in RA patients, the dosing interval might also be increased thus eliminating the need for daily injections.

REFERENCES

1. S. P. Eisenberg, R. J. Evans, and W. P. Arend. Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. *Nature.* **343**:341–5 (1990).
2. G. V. Campion, M. E. Lebsack, J. Lookabaugh, G. Gordon, M. Catalano, and The IL-1ra arthritis study group. Dose-range and dose-frequency study of recombinant interleukin-1 receptor antagonist in patients with rheumatoid arthritis. *Arthritis Rheum.* **39**:1092–1101 (1996).
3. D. E. Trentham, A. S. Townes, and A. H. Kang. Autoimmunity to type II collagen: an experimental model of arthritis. *J. Exp. Med.* **146**:857–868 (1977).
4. K. Terato, R. Hashida, K. Miyamoto, T. Morimoto, Y. Kata, S. Kobayashi, T. Tajima, S. Otake, H. Homi, and Y. Nagai. Histologi-

cal, immunological, and biochemical studies on type II collagen arthritis in rats. *Biomed. Res.* **3**:495–523 (1982).
5. T. Maack, V. Johnson, S. T. Kau, J. Figueiredo, and D. Sigulem. Renal filtration, transport, and metabolism of low-molecular-weight proteins: a review. *Kidneys International* **16**:251–270 (1979).
6. N. E. Larsen and E. A. Balazs. Drug delivery systems using hyaluronan and its derivatives. *Adv. Drug Delivery Rev.* **7**:279–293 (1991).
7. R. Cortivo, P. Brun, A. Rastrelli, and G. Abatangelo. *In vitro* studies on biocompatibility of hyaluronic acid esters. *Biomaterials* **2**:727–730 (1991).
8. N. E. Larsen, C. T. Pollak, K. Reiner, E. Leshchiner, and E. A. Balazs. Hylan gel biomaterial: dermal and immunologic compatibility. *J. Biomed. Materials Res.* **27**:1129–1134 (1993).
9. H. Bothner and D. Wik. Rheology of hyaluronate. *Acta Otolaryngol. (Stockholm) Suppl.* **442**:25–30 (1987).
10. J. A. Hunt, H. N. Joshi, V. J. Stella, and E. M. Topp. Diffusion and drug release in polymer films prepared from ester derivatives of hyaluronic acid. *J. Contr. Rel.* **12**:159–169 (1990).
11. L. Illum, N. F. Farraj, A. N. Fisher, I. Gill, M. Miglietta, and L. M. Bendetti. Hyaluronic acid microspheres as a nasal delivery system for insulin. *J. Contr. Rel.* **29**:133–141 (1994).
12. P. T. Prisell, O. Camber, J. Hiselius, and G. Norstedt. Evaluation of hyaluronan as a vehicle for peptide growth factors. *Int. J. Pharm.* **85**:51–56 (1992).
13. E. Ghezzo, L. M. Benedetti, M. Rochiva, F. Bivano, and L. Callagaro. Hyaluronic acid derivative microspheres as NGF delivery devices: preparation methods and *in vitro* release characterization. *Int. J. Pharm.* **87**:21–29 (1992).
14. J. Meyer, L. Whitcomb, M. Treuheit, and D. Collins. Sustained in vivo activity of recombinant human granulocyte colony stimulating factor (rHG-CSF) incorporated into hyaluronan. *J. Contr. Rel.* **35**:67–72 (1995).
15. C. A. Dinarello. Biologic basis for interleukin-1 in disease. *Blood* **87**:2095–2147 (1996).
16. W. P. Arend and J. P. Dayer. Inhibition of the production and effects of interleukin-I and tumor necrosis factor- in rheumatoid arthritis. *Arthritis Rheum.* **38**:151–160 (1995).
17. J. A. Eastgate, N. C. Wood, F. S. diGiovine, J. A. Symons, F. M. Grinlinton, and G. W. Duff. Correlation of plasma interleukin-I levels with disease activity in *rheumatoid arthritis. Lancet.* Sept. 24:706–709 (1988).
18. J. H. Schwab, S. K. Anderle, R. R. Brown, F. G. Dalldorf, and R. C. Thompson. Pro- and anti-inflammatory roles of interleukin-1 in recurrence of bacterial cell wall-induced arthritis in rats. *Infection Immunity* **59**:4436–4442 (1991).
19. L. A. B. Joosten, M. M. A. Helsen, F. A. J. van de Loo, and W. B. van den Berg. Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. *Arthritis Rheum.* **39**:797–809 (1996).