COMBINATION BENEFIT OF TREATMENT WITH THE CYTOKINE INHIBITORS INTERLEUKIN-1 RECEPTOR ANTAGONIST AND PEGylated SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TYPE I IN ANIMAL MODELS OF RHEUMATOID ARTHRITIS

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Objective. To determine the potential for additive or synergistic effects of combination therapy with the recombinant anticytokine agents interleukin-1 receptor antagonist (IL-1Ra) and PEGylated soluble tumor necrosis factor receptor type I (PEG sTNFRI) in established type II collagen–induced arthritis (CIA) and developing adjuvant-induced arthritis (AIA) in rats.

Methods. Rats with established CIA or developing AIA were treated with various doses of IL-1Ra in a slow-release hyaluronic acid vehicle or with PEG sTNFRI, either alone or in combination with the IL-1Ra. The effects of treatment were monitored by sequential caliper measurements of the ankle joints or hind paw volumes, final paw weights, and histologic evaluation with particular emphasis on bone and cartilage lesions.

Results. Combination therapy with IL-1Ra and PEG sTNFRI in rats with CIA resulted in an additive effect on clinical and histologic parameters when moderately to highly efficacious doses of each protein were administered. Greater-than-additive effects were seen when an inactive dose of IL-1Ra was given in combination with moderately to minimally active doses of PEG sTNFRI. Plasma levels associated with the latter effect (for both proteins) were similar to those seen in rheumatoid arthritis (RA) patients in clinical trials with these agents. Combination therapy in the AIA model generally resulted in additive effects, but some parameters showed a greater-than-additive benefit.

Conclusion. The results provide preclinical support for the hypothesis that IL-1Ra administered in combination with PEG sTNFRI might provide substantially more clinical benefit to RA patients than either agent alone at blood levels that are currently achievable in patients.

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of the joints with concomitant destruction of cartilage and bone. The involvement of cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), in the pathogenesis of RA is now well accepted as a result of numerous studies in animal models as well as in humans with the disease (1–11). The IL-1 receptor antagonist (IL-1Ra) is a specific receptor antagonist that competitively inhibits the binding of IL-1β and IL-1α to human and animal types I and II IL-1 receptors (12). Several clinical trials have been completed in which IL-1Ra has been administered long term to patients with RA (13,14). The results indicate that treatment with IL-1Ra lowers the levels of acute-phase proteins and the counts of swollen joints and may inhibit radiographic progression of disease (14,15). This protein has proved efficacious in various animal models of arthritis, both alone (10,11) and in combination with methotrexate (16), where the potential for additive effects was demonstrated.

Treatment with soluble TNF receptors (sTNFR) and antibodies to TNF has been shown to be clinically efficacious in RA patients (17–21). Animal models of arthritis in which these agents were evaluated predicted the excellent clinical response in humans (22–27). Several animal studies have focused on the efficacy of the high-affinity, monomeric PEGylated type I TNFR (PEG

TNFRI) administered alone (28) or in combination with other agents, such as methotrexate, dexamethasone, and indomethacin (29,30), where the potential for additive or synergistic effects was shown.

The purpose of the present study was to determine the potential benefit of combination treatment with the specific cytokine inhibitors IL-1Ra and PEG sTNFRI when given at dosages designed to achieve clinically relevant blood levels in order to support the clinical investigation of this approach.

MATERIALS AND METHODS

Animals. Female and male Lewis rats (175–225 gm; Charles River, Portage, MI) were used in these studies. Animals were allowed to acclimate for at least 7 days prior to initiation of experiments. Rats were housed in polycarbonate cages (2–4 per cage) and were allowed ad libitum access to food and water. All animal use was in accordance with the United States Department of Agriculture guidelines for humane care.

Materials. Recombinant IL-1Ra in hyaluronic acid (HA; 20 or 100 mg/ml) (10) and PEG recombinant sTNFRI (3, 1, or 0.3 mg/ml) (31) were produced at Amgen (Thousand Oaks, CA). Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Sigma (St. Louis, MO) and Difco (Detroit, MI), respectively. The synthetic adjuvant N,N-dioctylddecyl-N′,N-bis(2-hydroxyethyl)propanediamine (LA) was from BolderPath (Boulder, CO). Type II collagen was purchased from Elastin Products (Owensville, MO).

Induction and treatment of collagen-induced arthritis (CIA) and evaluation of clinical effects. Female rats (8 per group) were given intradermal/subcutaneous (SC) injections of bovine type II collagen (2 mg/ml in Freund's incomplete adjuvant) at a single site at the base of the tail and over the back at 2 sites (250 μl in divided doses) on day 0 and day 7. Arthritis onset occurred on days 12, 13, and 14; as rats developed disease, they were randomized to study groups. Treatment was initiated on the first day that clinical signs of arthritis were clearly visible, as evidenced by ankle joint swelling.

IL-1Ra in the sustained-release delivery system of HA and PEG sTNFRI in phosphate buffered saline (PBS) vehicle were given alone and in combination. Treatment with IL-1Ra (100 or 20 mg/kg) in HA was administered SC beginning on day 1 of arthritis and continuing through day 6. Treatment with PEG sTNFRI (3, 1, or 0.3 mg/kg) was given intraperitoneally (IP) in PBS on days 1, 3, and 5 of clinical arthritis. Vehicle-treated control rats were given HA (SC on days 1–6) or PBS (IP on days 1, 3, and 5).

Caliper measurements of ankle joint diameter were made prior to the onset of arthritis, on the day of randomization (day 1 of arthritis), and on each subsequent study day until termination of the study on day 7 of arthritis. At termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for determination of paw weights as another measure of inflammation. Hind paws and

knee joints were then collected into formalin for histopathologic evaluation.

Induction and treatment of adjuvant-induced arthritis (AIA) and evaluation of clinical effects. Male rats (5–7 per group) were given a single SC (base of tail) injection of 100 μl of Freund's complete adjuvant to which 5 mg/ml of LA had been added. In this model, systemic inflammatory disease occurs in various tissues, including the spleen and liver, as well as in most joints (32–34).

IL-1Ra in the sustained release delivery system of HA and PEG sTNFRI in PBS were given alone and in combination. Treatment with IL-1Ra (100 mg/kg) in HA was administered SC beginning on day 8 post–adjuvant injection and continuing through day 13. Treatment with PEG sTNFRI (3 or 1 mg/kg) in PBS was given IP on days 9, 11, and 13.

Caliper measurements of ankle joint width were made prior to the onset of arthritis, and then every other day until the study was terminated on day 15 post–adjuvant injection. Hind paw volumes and body weights were measured on days 9, 11, 12, 14, and 15. At termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for determination of paw weights as another measure of inflammation. Spleen and liver were trimmed of extraneous tissue and weighed. The hind paws and spleen were then collected into formalin for histopathologic evaluation.

Histopathology. Ankle joints (CIA and AIA) and knee joints (CIA only) were collected into 10% neutral buffered formalin and maintained for at least 24 hours prior to placement in SurgiPath Decalcifier I solution (Grayslake, IL) for ~1 week. When decalcification was complete, the digits were trimmed, and the ankle joint was transected in the longitudinal plane to give 2 approximately equal portions. Knee joints were transected in the frontal plane to give 2 approximately equal portions. These were processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin for general evaluation and with toluidine blue for specific evaluation of cartilage changes. Multiple sections were prepared to ensure that the distal tibia was present with both cortices and that abundant distal tibial medullary space was available for evaluation.

Ankles from rats with AIA were scored for inflammation and bone resorption according to the following criteria (0–5 scales) (34). For bone resorption, scores were 0 = normal, 1 = minimal—small areas of resorption in distal tibial trabecular or cortical bone, not readily apparent on low magnification, rare osteoclasts, 2 = mild—more numerous areas of resorption in distal tibial trabecular or cortical bone, not readily apparent on low magnification, osteoclasts more numerous, 3 = moderate—obvious resorption of medullary trabecular and cortical bone without full-thickness defects in the cortex, loss of some medullary trabeculae, lesion apparent on low magnification, osteoclasts more numerous, 4 = marked—full-thickness defects in cortical bone, often with distortion of profile of the remaining cortical surface, marked loss of medullary bone of the distal tibia, numerous osteoclasts, no resorption in smaller tarsal bones, and 5 = severe—full-thickness defects in cortical bone, often with distortion of profile of the remaining cortical surface, marked loss of medullary bone of the distal tibia, numerous osteoclasts, resorption also present in smaller tarsal bones.

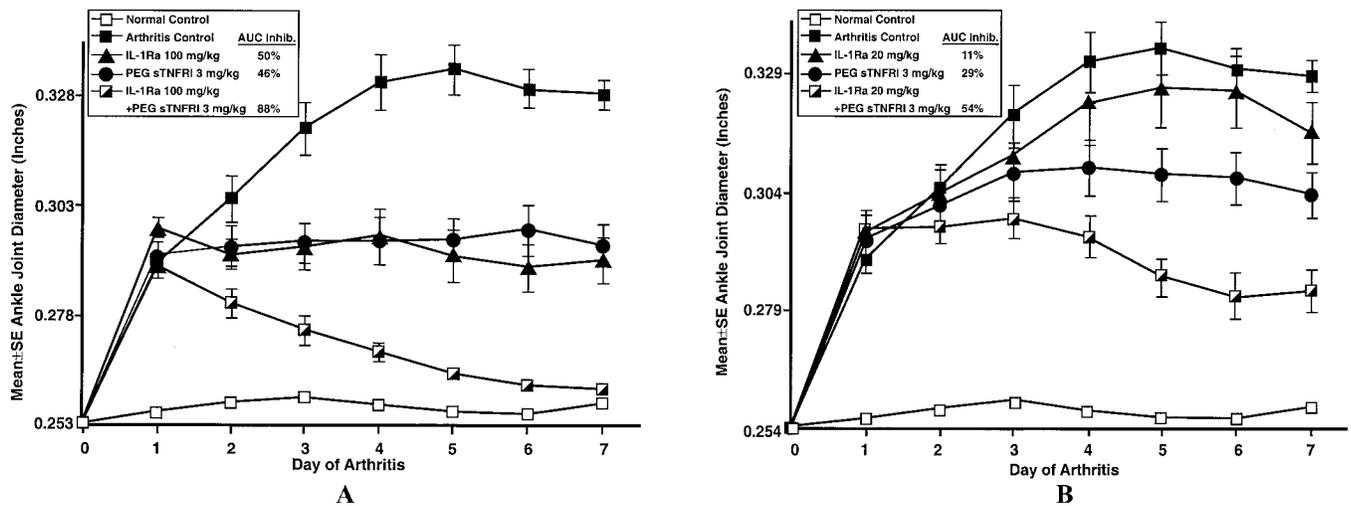For inflammation, scores were 0 = normal, 1 =

Figure 1. Changes in ankle joint diameter over time in rats with type II collagen–induced arthritis. **A,** Rats were treated with vehicles alone (hyaluronic acid [HA] for interleukin-1 receptor antagonist [IL-1Ra] subcutaneously [SC] every day and phosphate buffered saline for PEGylated soluble tumor necrosis factor α receptor type I [PEG sTNFRI] intraperitoneally [IP] every other day), with 100 mg/kg of IL-1Ra SC every day and vehicle IP every other day, with 3 mg/kg of PEG sTNF-RI IP every other day and HA SC every day, or with IL-1Ra and PEG sTNFRI in combination. The combination therapy produced additive benefits for ankle swelling, with the final measurements being similar to those of normal rats. **B,** Rats were treated with vehicles alone, with 20 mg/kg of IL-1Ra SC every day and vehicle IP every other day, with 0.3 mg/kg of PEG sTNFRI IP every other day and HA SC every day, or with the combination of IL-1Ra and PEG sTNFRI. Combination therapy produced additive benefits on ankle joint swelling over time. AUC = area under the curve; Inhib = inhibition; n = 8 rats per group.

minimal infiltration of inflammatory cells in periarticular tissue, 2 = mild infiltration, 3 = moderate infiltration with moderate edema, 4 = marked infiltration with marked edema, and 5 = severe infiltration with severe edema.

Cartilage damage was not scored in the AIA model because we have generally found this to be a minor feature and therefore not reliable for evaluation of potential treatment effects.

Histopathologic scoring for the tibiotarsal and knee joints of rats with CIA was similar to the inflammation and bone resorption scoring system used for rats with AIA. In addition, cartilage damage and pannus were scored because of the nature of the pathology in the CIA model. Cartilage damage was scored according to the following criteria (0–5 scale): 0 = normal, 1 = minimal-to-mild loss of toluidine blue staining with no obvious chondrocyte loss or collagen disruption, 2 = mild loss of toluidine blue staining with focal mild (superficial) chondrocyte loss and/or collagen disruption, 3 = moderate loss of toluidine blue staining with multifocal moderate (to middle-zone depth) chondrocyte loss and/or collagen disruption, 4 = marked loss of toluidine blue staining with multifocal marked (to deep-zone depth) chondrocyte loss and/or collagen disruption, and 5 = severe diffuse loss of toluidine blue staining with multifocal severe (to tidemark depth) chondrocyte loss and/or collagen disruption.

Spleens from rats with AIA were stained with hematoxylin and eosin and evaluated microscopically for inhibition of the classic AIA pathology (lymphoid atrophy, increased extramedullary hematopoiesis, and pyogranulomatous inflammation in the white pulp) (32).

The total histologic score comprises the composite total score of histologic parameters of inflammation, pannus formation, cartilage changes, and bone resorption (11,34).

Plasma IL-1Ra determination. Blood samples for determinations of plasma levels of IL-1Ra were collected from the tail veins of isoflurane-anesthetized rats at various times postdosing with 20 or 100 mg/kg of IL-1Ra in HA. Samples were analyzed using an enzyme-linked immunosorbent assay with an antibody to IL-1Ra prepared (R&D Systems, Minneapolis, MN). The sensitivity of the assay was 22 pg/ml.

Statistical analysis. Clinical data for ankle width and paw volumes in each model were analyzed by determining the area under the dosing curve (AUC), with subsequent application of Student's *t*-test to these values. Paw, spleen, and liver weights and histopathology parameters for each group (mean ± SEM) were analyzed for differences using Student's *t*-test.

RESULTS

Effects of combination therapy on established CIA. All animals had arthritis of similar severity at study inception, as evidenced by comparable mean ankle joint diameters on day 1, when randomization occurred and treatment was initiated (Figures 1A and B). Rats given daily doses of 100 mg/kg of IL-1Ra in HA had good inhibition of paw swelling over time (expressed as the AUC) and final paw weights, while those treated with 20 mg/kg IL-1Ra in HA had minimal beneficial effects on these clinical parameters (Table 1).

Table 1. Summary of data from rats with CIA treated with IL-1Ra and PEG sTNFRI alone and in combination*

Treatment group	Body weight change (gm)	Hind paw weight			Ankle diameter, AUC for % inhibition from arthritis control	Composite total histologic score			
						Ankle		Knee	
		Absolute (gm)	AUC for swelling (gm)	Final, % inhibition from arthritis control		Total (gm)	% inhibition from arthritis control	Total (gm)	% inhibition from arthritis control
Normal control rats	5.41 ± 0.95†	1.317 ± 0.012†	1.293 ± 0.004†	100.0	100.0	0.0 ± 0.0†	100.0	0.0 ± 0.0†	100.0
Rats with CIA									
HA + vehicle control IP/SC	−23.23 ± 3.4	1.850 ± 0.036	1.632 ± 0.025	–	–	14.0 ± 0.7	–	7.8 ± 1.2	–
Vehicle + IL-1Ra									
100 mg/kg	−15.88 ± 2.4	1.495 ± 0.045†	1.462 ± 0.025†	66.0	50.0	8.0 ± 1.1†	43.0	0.9 ± 0.6†	88.0
20 mg/kg	−18.16 ± 2.6	1.756 ± 0.060	1.595 ± 0.038	17.0	11.0	13.4 ± 1.3	4.0	5.8 ± 1.3	26.0
HA + PEG sTNFRI									
3 mg/kg	−13.11 ± 4.36	1.602 ± 0.048†	1.477 ± 0.024†	46.0	46.0	8.8 ± 1.2†	37.0	6.4 ± 1.1	18.0
1 mg/kg	−16.70 ± 4.9	1.653 ± 0.041†	1.507 ± 0.022†	36.0	37.0	9.9 ± 1.3†	29.0	6.9 ± 1.1	12.0
0.3 mg/kg	−18.51 ± 3.25	1.700 ± 0.039†	1.533 ± 0.026†	28.0	29.0	11.0 ± 1.0†	21.0	7.9 ± 1.4	0.0
IL-1Ra 100 mg/kg + PEG sTNFRI									
3 mg/kg	−7.69 ± 3.06†	1.346 ± 0.017†	1.342 ± 0.009†	93.0	88.0	1.8 ± 0.4†	87.0	0.0 ± 0.0†	100.0
1 mg/kg	−8.99 ± 2.2†	1.340 ± 0.013†	1.371 ± 0.008†	94.0	77.0	2.3 ± 0.5†	84.0	0.0 ± 0.0†	100.0
0.3 mg/kg	−5.54 ± 3.04†	1.388 ± 0.022†	1.392 ± 0.014†	88.0	71.0	2.6 ± 0.5†	81.0	0.0 ± 0.0†	100.0
IL-1Ra 20 mg/kg + PEG sTNFRI									
3 mg/kg	−6.46 ± 3.56†	1.387 ± 0.036†	1.395 ± 0.018†	86.0	70.0	3.1 ± 0.7†	78.0	0.0 ± 0.0†	100.0
1 mg/kg	−1.43 ± 2.84	1.421 ± 0.042†	1.429 ± 0.020†	79.0	60.0	4.4 ± 0.7†	69.0	0.2 ± 0.1†	97.0
0.3 mg/kg	−8.38 ± 3.4†	1.485 ± 0.041†	1.449 ± 0.020†	67.0	54.0	5.4 ± 0.9†	61.0	1.0 ± 0.3†	87.0

* Paw weight represents the final paw weight (in grams). Composite total histologic score represents the scores for inflammation, pannus, cartilage damage, and bone resorption. All groups contained 8 rats each. CIA = (type II) collagen-induced arthritis; IL-1Ra = interleukin-1 receptor antagonist; PEG sTNFRI = PEGylated soluble tumor necrosis factor receptor type I; AUC = area under the curve (in inches); HA = hyaluronic acid; IP = intraperitoneal; SC = subcutaneous.
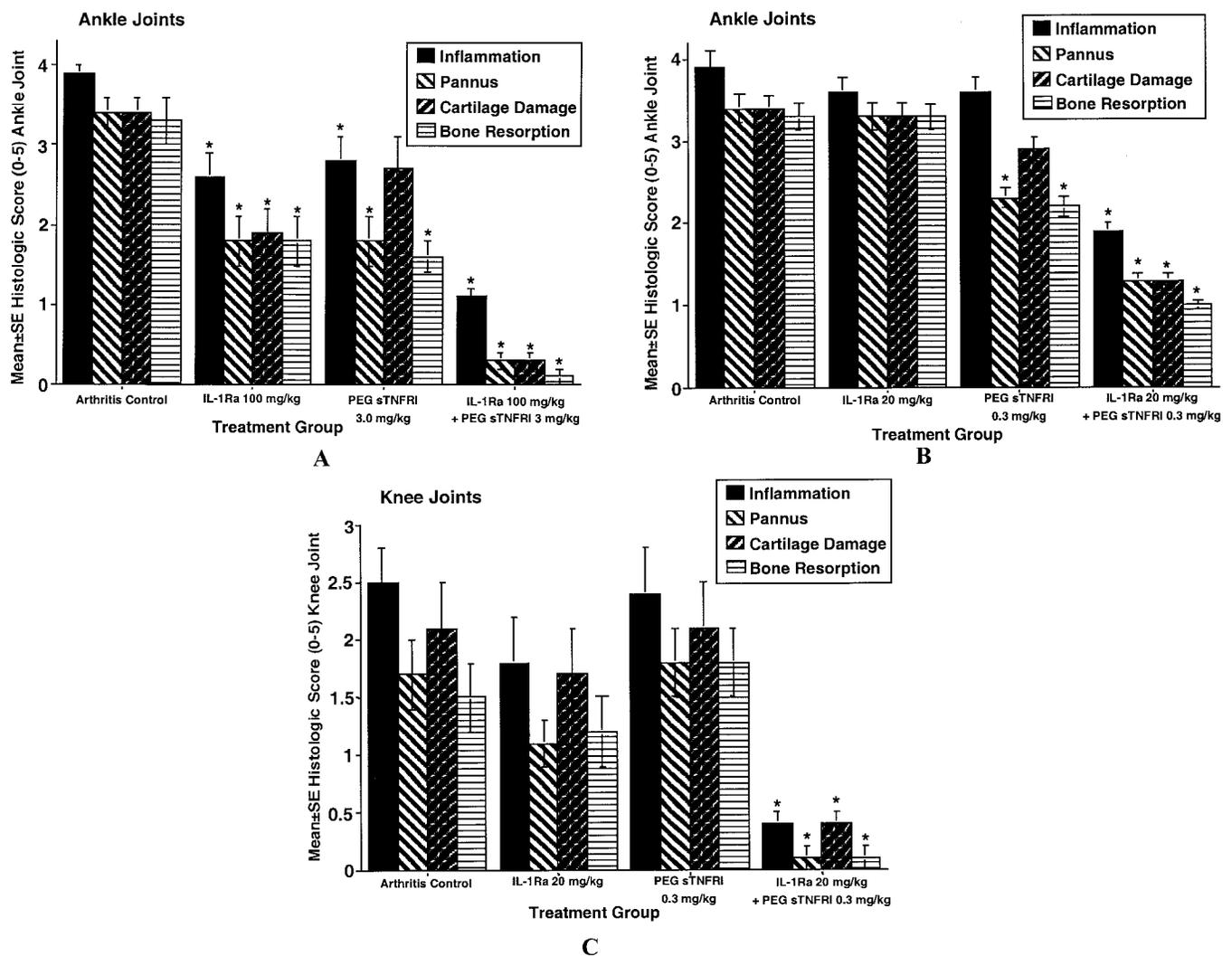† $P = 0.05$ versus vehicle control, by Student's 2-tailed t-test.

Figure 2. Changes in histologic parameters in the ankle joints (**A** and **B**) and knee joints (**C**) of rats with type II collagen–induced arthritis. **A,** Rats were treated with vehicles alone (HA for IL-1Ra SC every day and phosphate buffered saline for PEG sTNFRI vehicle IP every other day), with 100 mg/kg of IL-1Ra SC every day and vehicle IP every other day, with 3 mg/kg of PEG sTNFRI IP every other day and HA SC every day, or with IL-1Ra and PEG sTNFRI in combination. Combination therapy produced additive benefits on all parameters, resulting in dramatic inhibition of ankle joint pathology. **B,** Rats were treated with vehicles alone, with 20 mg/kg of IL-1Ra SC every day and vehicle IP every other day, with 0.3 mg/kg of sTNFRI IP every other day and HA SC every day, or with IL-1Ra and PEG sTNFRI in combination. Combination therapy produced greater-than-additive benefits on all parameters, resulting in good inhibition of ankle joint pathology. **C,** Rats were treated as in **B,** at the same dosages and protocol. Combination therapy produced greater-than-additive benefits on all parameters, resulting in excellent inhibition of knee joint pathology. * = $P \leq 0.05$ versus control, by Student's 2-tailed t-test; n = 8 rats per group. See Figure 1 for definitions.

Microscopic evaluation of joints revealed good inhibition of ankle pathology and excellent inhibition of knee lesions in rats treated with 100 mg/kg of IL-1Ra. The magnitude of inhibition of cartilage and bone lesions was generally similar at this dosage (Figures 2A–C). Treatment with IL-1Ra at 20 mg/kg had little beneficial effect on histologic parameters in the ankle

and the knee joints (Table 1). There was no beneficial effect on body weight gain with either dose of IL-1Ra (Table 1).

Treatment with PEG sTNFRI (3, 1, or 0.3 mg/kg) resulted in dose-responsive inhibition of the AUC for paw swelling, final paw weights, and total histologic scores for ankle joints (Table 1). Knee joint pathology
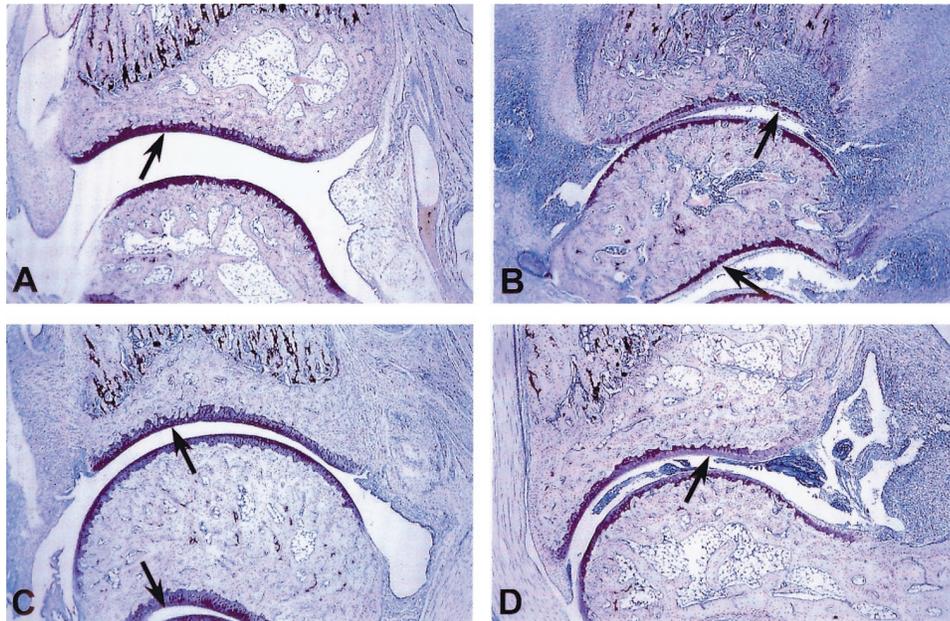
Figure 3. Photomicrographs of toluidine blue–stained ankle joints from rats with type II collagen–induced arthritis. **A,** Normal control rat, showing intense staining of normal articular cartilage **(arrow)** and absence of infiltrate in the synovium, **B,** Arthritic, vehicle-treated control rat, showing severe infiltration of inflammatory cells into the synovium and markedly diminished overall toluidine blue staining of the cartilage, as well as pannus formation and destruction **(arrows)** of cartilage and subchondral bone. **C,** Arthritic rat treated with the combination of IL-1Ra 100 mg/kg and PEG sTNFRI 3 mg/kg, showing largely intact **(arrows)** articular cartilage and subchondral bone, with mild inflammatory cell infiltration into the synovium. **D,** Arthritic rat treated with IL-1Ra 20 mg/kg and PEG sTNFRI 0.3 mg/kg, showing mild loss of proteoglycan from the articular cartilage, as evidenced by diminished toluidine blue staining. However, the collagenous portion of the cartilage is largely intact **(arrow)**, and there is little evidence of subchondral bone resorption. The synovium has moderate inflammatory cell infiltration. See Figure 1 for definitions.

was not inhibited significantly by any dosage of PEG sTNFRI (Table 1). The magnitude of inhibition of bone resorption was consistently greater than that of cartilage damage at all doses of PEG sTNFRI in both the knee and the ankle joints. There was no beneficial effect on body weight gain with any dose of PEG sTNFRI alone.

Combination therapy with IL-1Ra 100 mg/kg and PEG sTNFRI (all doses) resulted in additive beneficial effects on the AUC for paw swelling and final paw weights, with all combinations resulting in excellent amelioration of the clinical signs of arthritis (Table 1). In addition, significant benefit was also observed on body weight gain (Table 1). Additive effects on ankle swelling (resulting in 88% inhibition of the AUC) over time were found for the combination of 100 mg/kg IL-1Ra and 3 mg/kg PEG sTNFRI (Figure 1A). Additive effects were generally seen on the histologic parameters, except that rats treated with the combination of 100 mg/kg IL-1Ra

and 0.3 mg/kg PEG sTNFRI had greater-than-additive effects on the histologic scores in both the ankle and the knee (Table 1). Additive effects on histologic parameters for the combination of 100 mg/kg IL-1Ra and 3 mg/kg PEG sTNFRI are shown in Figure 2A.

Combination therapy with IL-1Ra 20 mg/kg and PEG sTNFRI (all doses) resulted in greater-than-additive beneficial effects on the AUC for paw swelling and the final paw weights, with all combinations resulting in good-to-excellent amelioration of the clinical signs of arthritis (Table 1). In addition, significant benefit on body weight gain (Table 1) was also observed. Additive effects on ankle swelling (resulting in 54% inhibition of the AUC) over time are shown for the combination of 20 mg/kg IL-1Ra and 0.3 mg/kg PEG sTNFRI in Figure 1B. Effects seen on the histologic parameters with these combinations were much greater than additive and were excellent in all cases (Table 1). Greater-than-additive

Table 2. Summary of data from rats with AIA treated with IL-1Ra and PEG sTNFRI alone or in combination*

Treatment group	Body weight change	Paw weight		Spleen weight		Liver weight		Histology			
								Bone resorption		Inflammation	
		Absolute weight, mean ± SEM	Final, % inhibition from arthritis control	Relative weight, mean ± SEM	Final, % inhibition from arthritis control	Relative weight, mean ± SEM	Final, % inhibition from arthritis control	Score, mean ± SEM	% inhibition from arthritis control	Score, mean ± SEM	% inhibition from arthritis control
Normal control rats, IP/SC	91.2 ± 0.81	1.813 ± 0.009	100	0.207 ± 0.005	100	5.706 ± 0.120	100	0 ± 0	100	0 ± 0	100
Rats with AIA											
HA + vehicle control IP/SC	29.8 ± 2.01	3.132 ± 0.081	0	0.572 ± 0.036	0	7.119 ± 0.281	0	3.07 ± 0.27	0	3.21 ± 0.21	0
IL-1Ra 100 mg/kg SC	42.2 ± 2.01	2.952 ± 0.060	14.0	0.481 ± 0.043	25	6.996 ± 0.188	9	1.75 ± 0.43†	43	2.5 ± 0.23†	22
PEG sTNFRI											
3 mg/kg IP	40.9 ± 2.23	2.671 ± 0.035†	35.0	0.413 ± 0.016†	44	6.498 ± 0.182	44	1.36 ± 0.25†	56	1.86 ± 0.18†	42
1 mg/kg IP	36.7 ± 2.98	2.825 ± 0.069†	23.0	0.398 ± 0.021†	48	6.414 ± 0.061†	50	2.14 ± 0.46	30	2.64 ± 0.27	18
sTNFRI 100 mg/kg SC + IL-1Ra											
3 mg/kg IP	46.9 ± 4.04	2.279 ± 0.033†	65.0	0.348 ± 0.016†	61	5.991 ± 0.142†	80	0 ± 0†	100	1.0 ± 0†	69
1 mg/kg IP	46.5 ± 2.64	2.583 ± 0.038†	42.0	0.362 ± 0.012†	58	6.110 ± 0.159†	71	1.21 ± 0.32†	60	1.71 ± 0.19†	47

* All groups contained 8 rats each. AIA = adjuvant-induced arthritis; IL-1Ra = interleukin-1 receptor antagonist; PEG sTNFRI = PEGylated soluble tumor necrosis factor receptor type I; IP = intraperitoneal; SC = subcutaneous; HA = hyaluronic acid.
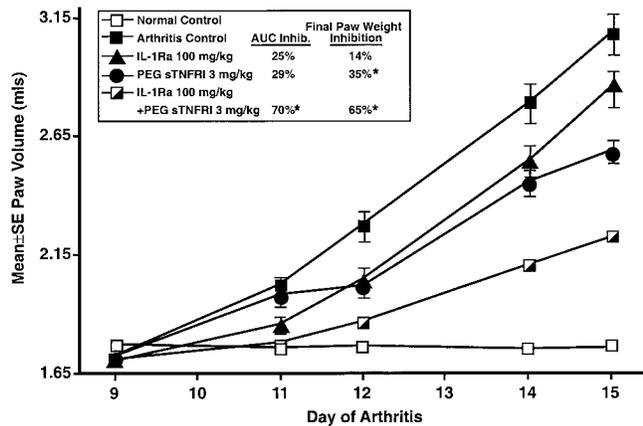† $P = 0.05$ versus vehicle control, by Student's 2-tailed t-test.

Figure 4. Changes in ankle paw volume over time in rats with adjuvant-induced arthritis treated with vehicles alone (HA for IL-1Ra SC every day and phosphate buffered saline for PEG sTNFRI IP every other day), with 100 mg/kg of IL-1Ra SC every day and vehicle IP every other day, with 3 mg/kg of PEG sTNFRI IP every other day and HA SC every day, or with IL-1Ra and PEG sTNFRI in combination. Combination therapy produced additive to slightly greater than additive benefits on ankle swelling over time. * = $P \leq 0.05$ versus control, by Student's 2-tailed t-test; n = 8 rats per group. See Figure 1 for definitions.

effects on histologic parameters for the combination of 20 mg/kg IL-1Ra and 0.3 mg/kg PEG sTNFRI are shown in Figures 2B and C, as well as in Figure 3.

Effects of combination therapy on developing AIA. Treatment with IL-1Ra alone resulted in minimal inhibition of ankle joint swelling in rats with AIA. Treatment with 3 mg/kg PEG sTNFRI resulted in 35% inhibition of final paw weights (Table 2 and Figure 4). Combination therapy yielded 70% inhibition of paw swelling over time (expressed as the AUC) and 65% inhibition of final paw weights. Combination benefit was also seen on inhibition of inflammation in the spleen and liver, as assessed by the weights of these organs (Table 2). Histopathologic evaluation of the spleen confirmed that the beneficial effects of treatment on spleen weights were associated with a return to normal morphology in animals given the combination therapy (results not shown). Body weight change associated with AIA showed modest benefit with either treatment alone and mildly increased benefit with combination treatment (data not shown).

Histologic parameters of inflammation and bone resorption were moderately decreased with either treatment alone and dramatically decreased when the treatments were administered in combination (Figure 5 and Table 2). Administration of a lower dose of PEG sTNFRI (1 mg/kg) in combination with IL-1Ra also

resulted in additive effects on the various parameters (Table 2).

Plasma levels of IL-1Ra in rats treated with 20 or 100 mg/kg of IL-1Ra in HA. Peak levels of IL-1Ra after a single SC injection of 100 mg/kg were 9 μg/ml at 6 hours postdosing (Figure 6) and fell below 1 μg/ml 24 hours after injection. Peak levels of IL-1Ra after a single SC injection of 20 mg/kg were 2 μg/ml at 3 hours postdosing (Figure 6) and fell below 0.08 μg/ml 24 hours after injection.

DISCUSSION

The findings of the present study demonstrate that combination therapy with higher, more efficacious doses of IL-1Ra and PEG sTNFRI results in additive effects in rats with established CIA. Minimally effective doses of PEG sTNFRI (0.3 mg/kg) in combination with ineffective doses of IL-1Ra (20 mg/kg) result in much greater than additive effects on all parameters and excellent overall inhibition of established arthritis.

IL-1 appears to be an important mediator of CIA in rats. In rats treated with daily doses of 100 mg/kg of IL-1Ra in HA, 50% inhibition (by AUC) to 66% inhibition (by paw weight) of clinical parameters of estab-
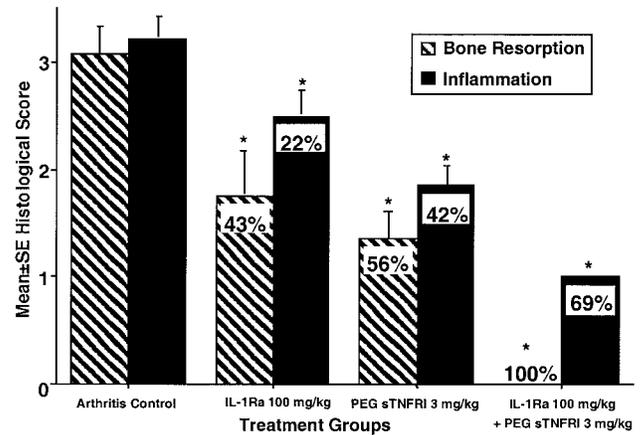

Figure 5. Changes in histologic parameters of inflammation and bone resorption in rats with adjuvant-induced arthritis treated with vehicles alone (HA for IL-1Ra SC every day and phosphate buffered saline for PEG sTNFRI IP every other day), with 100 mg/kg of IL-1Ra SC every day and vehicle IP every other day, with 3 mg/kg of PEG sTNFRI IP every other day and HA SC every day, or with IL-1Ra and PEG sTNFRI in combination. Combination therapy produced additive benefits on inflammation and bone resorption, resulting in 100% inhibition of the aggressive bone resorption that occurs in this disease and excellent inhibition of periarticular inflammation. * = $P \leq 0.05$ versus controls, by Student's 2-tailed t-test; n = 8 rats per group. See Figure 1 for definitions.
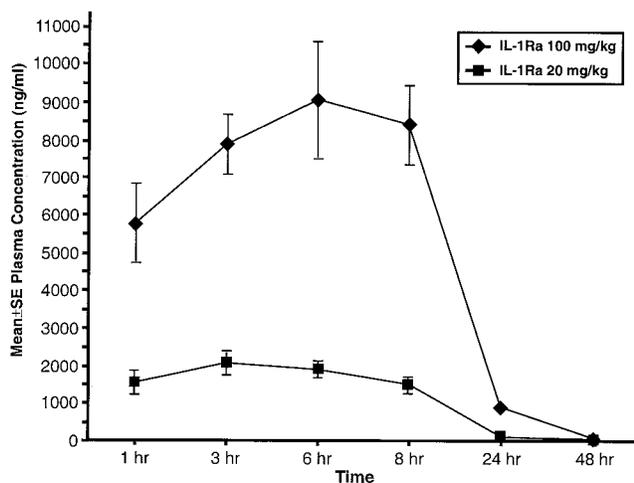
Figure 6. Plasma concentrations of interleukin-1 receptor antagonist (IL-1Ra) in rats treated with single subcutaneous doses of 20 mg/kg or 100 mg/kg in the slow-release vehicle hyaluronic acid.

lished arthritis was achieved. Histologic changes in knee joints were dramatically suppressed (88%) at this dosage. This dosing regimen results in blood levels of 6–9 μg/ml for 1–8 hours after the dose is administered, with the blood levels falling to 0.875 μg/ml at 24 hours, when the next dose is due. Although administration of IL-1Ra in HA dramatically improves the pharmacokinetic profile over that seen with aqueous vehicles (10), continuous-infusion studies in which blood levels are maintained at ~5 μg/ml have shown even greater inhibition of this established arthritis (11).

Since it is unlikely that similar continuously high blood levels would be achieved clinically, we chose to perform these combination studies using a regimen that more closely approximates the pharmacokinetic profile seen in humans given 2 mg/kg of IL-1Ra (in its current aqueous vehicle), which results in approximate levels of 1.2–1.6 μg/ml at 12 hours, 0.8 μg/ml at 18 hours, and 0.2 μg/ml at 24 hours, when the next dose would be given (Bendele A: unpublished observations). The blood levels in rats treated with 100 mg/kg IL-1Ra in HA are much higher (especially peak levels) than those seen in humans given 1–2 mg/kg. Blood levels (for the first 8 hours postdosing) in rats given 20 mg/kg are similar to those in humans in current clinical trials who are receiving daily doses of 1–2 mg/kg, but trough levels at 24 hours are much lower than those in humans. Therefore, the data from this study of combination therapy demonstrate efficacy in rodent models at blood levels of IL-1Ra that are much higher than, as well as blood levels that are comparable to, those achieved with the 1–2 mg/kg

dose currently being utilized in monotherapy trials with this agent, in which efficacy has been demonstrated (11,14,15).

TNFα also appears to be an important mediator of established CIA in rats. Forty-six percent inhibition (by AUC and paw weight) of clinical parameters of established arthritis was achieved when rats were treated every other day with 3 mg/kg doses of PEG sTNFRI. This dosing regimen results in peak blood levels of 6.8 μg/ml at 24 hours, with the levels falling to 4.2 μg/ml at 48 hours, when the next dose is due (28). These blood levels are high compared with those being achieved in early phase I trials of this agent in humans (35). However, the peak blood levels in rodents given 0.3 mg/kg (0.5 μg/ml) to 1 mg/kg (2.5 μg/ml) every other day are comparable to the peak levels in humans treated with similar weekly doses of PEG sTNFRI (28,35,36). Efficacy data are not currently available for PEG sTNFRI; however, results of a previous trial with a dimeric PEG construct in which efficacy was measured suggested that average blood levels of ~0.6 μg/ml were associated with swollen joint counts that were 55% of baseline (21).

Therefore, this study of combination therapy in rats with established CIA demonstrates efficacy in association with blood levels of both biologic agents that range from higher to lower than those that are currently being achieved in humans, thus providing a full range of potential scenarios. When both agents are given together at the highest dosages (100 mg/kg of IL-1Ra and 3 mg/kg of PEG sTNFRI), near-total suppression of this aggressive established arthritis occurs, with the effects being additive compared with either agent alone. The combinations of 100 mg/kg of IL-1Ra and either 1 mg/kg or 0.3 mg/kg of PEG sTNFRI (producing blood levels that are reasonably close to those seen in humans, especially with PEG sTNFRI), resulted in excellent additive (1 mg/kg) to greater-than-additive (0.3 mg/kg, histologic parameters only) effects on all aspects of CIA. When IL-1Ra was given at a completely inactive dosage of 20 mg/kg (which results in blood levels that are much lower than the levels currently achieved in humans) in combination with the clinically relevant dosages of 1 mg/kg or 0.3 mg/kg of PEG sTNFRI, patterns of efficacy were consistently greater than additive for all parameters. These data suggest a potential for synergistic clinical effects at dosages of IL-1Ra that are lower than those currently used and at dosages of PEG sTNFRI potentially lower than the dosages that have been proposed.

Evaluation of efficacy in the rat model of developing AIA utilized dosages of 100 mg/kg of IL-1Ra and

either 3 mg/kg or 1 mg/kg of PEG sTNFRI. Additive to greater-than-additive effects were seen for all parameters with both combinations.

Although there are no human trials to date, the combination of 2 anticytokines, such as IL-1Ra and an sTNFR, may offer greater efficacy than either agent alone (37,38). In a previously published animal study, the combination of IL-1Ra with a dimeric TNFRI (TNFRI p55), TNF binding protein (21), or an anti-TNFα antibody significantly reduced disease activity in a murine model of streptococcal cell wall–induced arthritis (39).

Further insight into the role of TNFα and IL-1 in inflammation and cartilage destruction has emerged from studies of experimental arthritis. In murine models, using zymosan, immune complexes, or T cell allergens as arthritogenic stimuli, it was shown that cartilage destruction was highly dependent on IL-1, whereas TNFα involvement was limited (9,40). Finally, in a recently published study, the effects of neutralization of either TNFα or IL-1 on joint structures in established CIA in the murine model were studied (41). Both treatment with soluble TNF binding protein and treatment with anti–IL-1 ameliorated disease activity when administered shortly after the onset of CIA. Serum analysis revealed that early treatment with anti-TNFα did not decrease the disease activity in the cartilage, as indicated by the elevated levels of cartilage oligomeric matrix protein (41).

The biologic activities of IL-1 are synergistic with other cytokines and growth factors; however, the synergism of IL-1 plus TNFα is highly consistent and has been frequently reported. The synergism between IL-1 and TNFα is often observed in vivo (42,43), whereas the synergism between IL-1 and various growth factors relates mostly to cytokine production and prostanoid synthesis and is primarily an in vitro finding (43). The mechanism for synergy may involve receptor modulation, but TNF receptors are down-regulated by IL-1 (44,45). Synergism may also be explained at the level of signal transduction (46). Since the signaling mechanism of IL-1 and TNFα appear to be similar, additive rather than synergistic effects should be observed. Part of the synergism in vivo may be explained by the ability of TNFα to induce IL-1 and vice versa (47). For example, during heat-killed *Staphylococcus epidermidis*–induced shock in rabbits, IL-1Ra administration reduced circulating levels of TNFα (48), suggesting that endogenous IL-1 induces TNFα. In baboons with *Escherichia coli*–induced shock, anti-TNFα treatment reduced circulating levels of IL-1β (49).

At present, there is no single molecular mechanism that would explain the synergism of IL-1 and TNFα. However, this synergism may explain why the combination of IL-1Ra plus sTNFR was more effective in blocking disease in animal studies than either strategy alone. These findings require confirmation in humans.

The basis for attributing part of the success of TNFα neutralization in RA to a decrease in the production of bioactive IL-1 can be found in a classic paper by Brennan et al (50). In that study, mixed cells from patients' synovial fluid or synovial tissues were cultured in the presence of neutralizing antibodies to human TNFα or lymphotoxin. Anti-TNFα dramatically reduced the spontaneous production of IL-1 activity, whereas anti-lymphotoxin did not (51). In RA patients treated with anti-TNFα agents, the reduction in circulating IL-1β confirms Brennan's observation that IL-1 production in RA is under the control of TNFα. However, it would not be unexpected that some of the TNFα production in RA is also under the control of IL-1.

Our results in 2 well-established animal models of arthritis that have been reasonably predictive of clinical efficacy (34), using dosing protocols that result in realistic blood levels with respect to clinical applicability, support the clinical investigation of combination therapy with the specific cytokine inhibitors IL-1Ra and PEG sTNFRI in RA patients.

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