CMLS, Cell. Mol. Life Sci. 57 (2000) 1457–1470
1420-682X/00/101457-14 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Anti-interleukin-1 and anti-tumor necrosis factor-α synergistically inhibit adjuvant arthritis in Lewis rats[1]

U. Feige[a,*], Y.-L. Hu[a], J. Gasser[a], G. Campagnuolo[a], L. Munyakazi[b] and B. Bolon[a]

[a]Department of Pharmacology/Pathology, Amgen, One Amgen Center Drive, M/S 15-2-A, Thousand Oaks (California 91320-1789, USA), Fax + 1 805 499 75 06, e-mail: ufeige@amgen.com
[b]Department of Biostatistics, Amgen, Thousand Oaks (California 91320-1789, USA)

Received 11 May 2000; received after revision 17 July 2000; accepted 19 July 2000

Abstract. Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) play dominant roles in mediating the progression of many inflammatory joint diseases, including rheumatoid arthritis in humans, collagen-induced arthritis in mice and rats, and adjuvant arthritis in rats. Blockade of either cytokine partially controls these diseases. The present study investigated the value of combination anti-cytokine therapy in arthritis: the efficacy of IL-1 receptor antagonist (IL-1ra) and 30 kDa polyethylene glycol (PEG)-conjugated soluble TNF receptor type I (PEG sTNF-RI) given together was assessed in Lewis rats with adjuvant arthritis. Administration of either IL-1ra or PEG sTNF-RI partially alleviated joint inflammation, loss of bone mineral density, and loss of body weight. In contrast, combination of these anti-cytokine treatments exhibited a synergistic capacity to inhibit these changes, even when combining doses of IL-1ra and PEG sTNF-RI that did not affect lesion severity when used alone. Statistical analysis of these adjuvant arthritis data using the isobologram method proved that IL-1ra and PEG sTNF-RI were clearly synergistic in inhibiting inflammation, loss of bone mineral density, loss of body weight, and histopathologic parameters of inflammation and joint destruction. These results suggest that treating autoimmune arthritic diseases with combinations of anti-IL-1 and anti-TNF molecules will achieve superior efficacy compared to the use of a single class of anti-cytokine agent and may allow for dose reductions that could prove useful in minimizing potential side effects.

Key words. Rheumatoid arthritis; interleukin 1; tumor necrosis factor; cytokine; rat; adjuvant arthritis; IL-1ra; soluble receptor.

In arthritic diseases, a plethora of pro- and anti-inflammatory cytokines is released [for reviews see refs 1–4]. The balance is believed to be shifted in favor of pro-inflammatory cytokines that drive the arthritic process [3, 4]. Studies with synovial explant cultures from rheumatoid arthritis (RA) patients have shown that interleukin (IL)-1 and tumor necrosis factor (TNF)-α are the dominant pro-inflammatory cytokines in this network [5, 6]. Inhibition of either IL-1 or TNF-α reduces the extent of inflammation both in RA [7–14] and in various experimental models of arthritis in animals [15–25]. Therefore, administration of biological response-modifying agents to inhibit the action of pro-inflammatory cytokines is gaining rapid acceptance as a modality for early, aggressive treatment of RA [26]. In conventional rheumatology practice, anti-arthritic drugs are used in combination to simultaneously restrain multiple path-

* Corresponding author.
[1] Presented in part at the 63rd Annual Meeting of the American College of Rheumatology, Boston, USA 13–17 November 1999 [57].

Table 1. Study design.

Group	Treatment	Dose	Route	Schedule
1	normal control	–	–	–
2	AdA control	–	–	–
3	PEG sTNF-RI	0.25 mg/kg per day	SC injection	daily for 7 days
4	PEG sTNF-RI	1 mg/kg per day	SC injection	daily for 7 days
5	PEG sTNF-RI	4 mg/kg per day	SC injection	daily for 7 days
6	IL-1ra	0.2 mg/kg per hour	SC pump	7-day infusion
7	IL-1ra	1 mg/kg per hour	SC pump	7-day infusion
8	IL-1ra	5 mg/kg per hour	SC pump	7-day infusion
9	IL-1ra	0.2 mg/kg per hour	SC pump	7-day infusion
	PEG sTNF-RI	0.25 mg/kg per day	SC injection	daily for 7 days
10	IL-1ra	0.2 mg/kg per hour	SC pump	7-day infusion
	PEG sTNF-RI	1 mg/kg per day	SC injection	daily for 7 days
11	IL-1ra	1 mg/kg per hour	SC pump	7-day infusion
	PEG sTNF-RI	0.25 mg/kg per day	SC injection	daily for 7 days
12	IL-1ra	1 mg/kg per hour	SC pump	7-day infusion
	PEG sTNF-RI	1 mg/kg per day	SC injection	daily for 7 days
13	IL-1ra	5 mg/kg per hour	SC pump	7-day infusion
	PEG sTNF-RI	4 mg/kg per day	SC injection	daily for 7 days

Doses of IL-1ra and PEG sTNF-RI were selected based on previous studies (data not shown) to cover the range of the dose-response curve from (almost) inactive doses to fully active doses. Control groups with vehicle treatment(s) or implantation of osmotic minipumps with vehicle were not included in this study because prior studies in our laboratory had indicated no impact of these treatments on the development or severity of arthritis in this model (data not shown). The small group size ($n = 6$) was suitable for this study because past experience with this severe arthritis model indicates that clinical and histopathological variability in untreated arthritic rats using our endpoints is sufficiently minimal [47, and Feige et al., unpublished data]. AdA, adjuvant arthritis; SC, subcutaneous.

ways that contribute to the pathogenesis of RA. Various IL-1 and TNF inhibitors are used in such clinical regimens, often in conjunction with methotrexate [27]. At present, combinatorial strategies in which both anti-IL-1 and anti-TNF molecules are employed simultaneously have not been attempted. However, investigating combination therapy with anti-cytokines seems logical.

Numerous studies indicate that IL-1 and TNF-α can act synergistically to promote inflammmation. For example, IL-1 and TNF-α exhibit synergistic activity in vivo to recruit leukocytes in rabbit joints [28]. In vitro, IL-1 and TNF-α act synergistically to induce production of plasminogen activator by human articular chondrocytes [29], or collagenase and prostaglandin E_2 [30, 31], IL-6 [32], or IL-8 [33] by human synovial fibroblasts. This synergism may result from pro-inflammatory feedback loops by which IL-1 induces TNF-α, and vice versa [34–36]. Therefore, to postulate that simultaneous inhibition of both cytokines may be more beneficial than modulation of either cytokine alone seems resonable. A number of published experimental studies which have co-administered anti-IL-1 and anti-TNF-α treatments appear to support this contention. For example, in a standard rodent endotoxemia model, IL-1 receptor antagonist (IL-1ra) or polyethylene glycol (PEG)-recombinant soluble TNF receptor type I $(rsTNF\text{-}RI)_2$ alone had to be given concurrently with the lipopolysaccharide (LPS) challenge to provide maximum protection against lethality. In contrast, therapy with IL-1ra and PEG-$(rsTNF\text{-}RI)_2$ combinations could ameliorate lethality even when given several hours after the LPS challenge [37]. In like manner, treatment with both IL-1ra and PEG-$(rsTNF\text{-}RI)_2$ resulted in superior reduction of clinical signs relative to either agent alone in rats with experimental autoimmune encephalomyelitis (EAE) [38]. In rodent ovariectomy models of estrogen-deficiency osteoporosis, simultaneous blockade of IL-1 and TNF-α was a requirement to completely prevent bone loss [39, 40]. Therefore, combination therapy with anti-IL-1 and anti-TNF agents to simultaneously reduce both cytokines could act synergistically to alter the clinical course of arthritic disease in both animal models and RA patients.

At present, the ability of cytokine inhibitors to protect against skeletal destruction as well as control inflammation in RA is generating considerable interest. Bone erosions, non-glucocorticoid-induced subchondral osteopenia, and systemic osteoporosis are clinical findings in RA [41, 42]. Similarly, in animal models of arthritis, both loss of bone mineral density [43–45] and frank erosions are observed. Most agents in the anti-arthritic armamentarium reduce the extent of inflammation but do not effectively modify the degree of bone damage. The protective ability afforded by cytokine-inhibiting agents has engendered an expanded search for additional anti-cytokine biological-response modifiers.

The principal objective of this study was to analyze the effects of combination treatment with anti-IL-1 plus anti-TNF-α agents in mycobacteria-induced adjuvant arthritis in Lewis rats. This model represents a systemic inflammatory condition in which damage to the affected joints is quite severe [46]. We assessed the ability of IL-1ra and PEG sTNF-RI, both alone and in tandem, to ameliorate the destructive nature of these arthritic lesions. Special attention was given to the nature of the efficacious outcome (i.e., additivity vs. synergism) induced by the combination treatment. Our data indicate a significant benefit in simultaneously blocking the pro-inflammatory signaling cascade at the level of both IL-1 and TNF in adjuvant arthritis in Lewis rats.

Material and methods

Animals. Male Lewis rats (Charles River, Wilmington, Mass.) were acclimated for 1 week. Prior to inception of the study, the rats' body weights had reached 180–200 g. They were randomly assigned to treatment groups (n = 6/group). Animals were given tap water and fed pelleted rodent chow (no. 8640; Harlan Teklad, Madison, Wis.) ad libitum; calcium and phosphorus contents were 1.2% and 1.0%, respectively. At necropsy, all animals were sacrificed by carbon dioxide inhalation. These studies were conducted in accordance with federal animal care guidelines and were pre-approved by the Amgen Institutional Animal Care and Use Committee (AIACUC).

Table 2. Histopathology grades for adjuvant arthritis

Inflammation	
0	normal
1	few inflammatory cells
2	mild inflammation
3	moderate inflammation (often but not always diffuse)
4	marked inflammation (diffuse and dense with synovial abscesses)
Skeletal remodeling (assesses both destruction and repair)	
0	normal
1	minimal marrow inflammation, and/or cartilage undermining ($\leq$two foci)
2	mild periosteal proliferation (usually tarsals), and/or marrow inflammation (tibia or tarsals), and/or cartilage undermining ($\geq$three foci)
3	moderate periosteal proliferation with minimal erosion of cortical bone (tibial metaphysis and/or tarsals)
4	marked periosteal and/or endosteal proliferation with extensive erosion of cortical bone of either tarsals OR (less frequently) tibia
5	marked periosteal and/or endosteal proliferation with full thickness erosion of cortical bone of tarsals AND tibia
Bone erosion (assesses destruction)	
0	normal
1	minimal loss of cortical or trabecular bone at a few sites
2	mild loss of cortical or trabecular bone at a modest number of sites (generally tarsals)
3	moderate loss of at many sites (usually the trabeculae of the tarsals, but sometimes the cortex of the distal tibia)
4	marked loss of bone at many sites (usually as extensive destruction of trabeculae in the tarsals, but sometimes with partial loss of cortical bone in the distal tibia)
5	marked loss of bone at many sites (with fragmenting of tarsal trabeculae AND full-thickness penetration of cortical bone in the distal tibia)

Induction and treatment of adjuvant arthritis

Adjuvant arthritis was induced on day 0 as described [47] by a single intradermal injection at the base of the tail of heat-killed *Mycobacterium tuberculosis* H37Ra (0.5 mg; Difco, Detroit, Mich.) suspended in 0.05ml paraffin oil (Crescent, Hauppauge, N.Y.). The clinical onset of arthritis was at day 9 as indicated by hind paw swelling and ambulatory difficulties. At this time, a 7-day course of therapy was initiated using the anti-cytokine agents (table 1). Groups of rats (n = 6) received recombinant human IL-1ra (anakinra; Amgen, Thousand Oaks, Calif.), which was administered at 0.2, 1, or 5 mg/kg per hour (4.8, 24, or 120 mg/kg per day, respectively) using implanted osmotic minipumps (2ML1; Alza, Palo Alto, Calif.). Pumps were implanted under isoflurane anesthesia in the dorsal subcutis on day 9, and wounds were sealed using steel clips. Additional groups of rats received recombinant human PEG sTNF-RI (Amgen) delivered once daily as a subcutaneous injection at 0.25, 1, or 4 mg/kg per day. Finally, groups of rats were treated with both agents at various dose combinations. All groups were run in parallel to limit variability associated with inter-study comparisons.

Experimental design. Inflammation, cachexia, periarthroidal osteoporosis, and erosion of bone and cartilage are the hallmarks of autoimmune arthritic diseases. We examined these sequelae using the following measurements: (i) paw volume for inflammation, (ii) body weight for cachexia, (iii) bone mineral density for osteoporosis, and (iv) histopathologic assessment to address erosions. These sensitive and quantifiable endpoints are well recognized for their ability to screen therapeutic candidates in experimental studies of arthritis, including the Lewis rat model of adjuvant arthritis.

Clinical assessment (body weight and mobility). Total body weights were recorded on days 0, 1, 4, and 8–16. On the same days, mobility was assessed qualitatively at cage side using a binary scale (normal or impaired) by noting the ability and propensity of animals to move about the cages. Motility was evaluated for the entire group rather than on an animal-by-animal basis.

Hind paw volume assessment (plethysmography). Onset and severity of inflammation were monitored by measuring the volume of hind paws using a refined volume displacement technique (plethysmography). Briefly, a

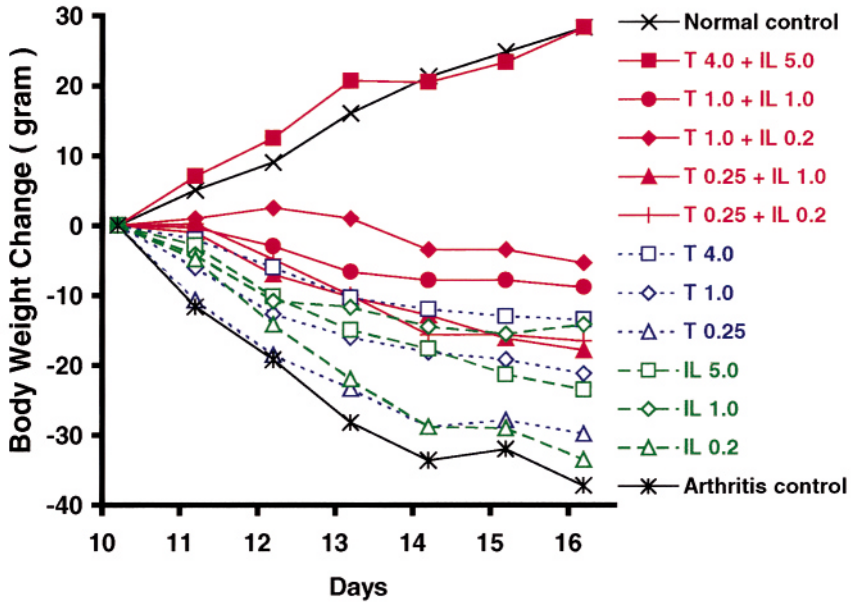

Figure 1. Inhibition of loss of body weight by combination treatment with IL-1ra and PEG sTNF-RI. Arthritis was induced in Lewis rats by injection of heat-killed mycobacteria in oil. From clinical onset of disease on day 9, groups of rats (n = 6) were treated through day 16 (IL = mg/kg per hour of IL-1ra; T = mg/kg per day of PEG sTNF-RI; see table 1 and Materials and methods). The figure shows changes in body weight relative to day 10. Bars represent the percent inhibition of body weight loss relative to that of the untreated arthritis control (Group 2, table 1). For clarity of the figure, error bars are omitted; for SEs and the results of the isobologram analysis see table 3.

100-ml Pyrex glass beaker containing about 100 ml of tap water (37 °C) with one drop of soap was placed on a balance (Model BP6100; Sartorius, Göttingen, Germany). The balance with the beaker was placed on a Jumbo Support Rack (VWR, San Francisco, Calif.) to allow adjustment in height such that the water surface was at eye level of the examiner. Balance sensitivity was set at an intermediate level (0.2; code 134 with BP6100) to control for subtle vibrations engendered by movement of the operator's hands or room air. With this optimal setting, differences between consecutive volume readings of the same paw were < 0.2 ml (data not shown). For each paw measurement, the balance was reset to zero (tara), and the rat's hind paw was dipped into the water to just above the tibiotarsal joint (hock, or ankle). A measurement was initiated by tipping a foot pedal. For each paw, two consecutive readings were taken by re-insertion of the paw into the re-tared beaker, after which the mean was calculated and used for further analysis. The measurement data were transmitted online via RS232 using WinWedge (TAL Technologies, Philadelphia, Penn.) to an Excel spreadsheet. Measurements were taken on day 0 (before induction of arthritis), 1, 4, and daily from day 8 to 16. Inhibition of paw inflammation was calculated based on the area under the curve (AUC) using the trapezoidal rule according to the formula

$$[1 - (\text{treated adjuvant arthritis} - \text{normal})/(\text{adjuvant arthritis} - \text{normal})] \times 100.$$

Bone densitometry. Dual-energy X-ray absorptiometry (DEXA) to measure bone mineral density has widespread application in human studies and has been adapted to rodents because of the need to examine bone integrity during longitudinal studies of rats [44, 45, 48–51]. At necropsy (day 16 post-immunization), hindpaws were removed at the fur line (just proximal to the hock) and stored in 70% ethanol until DEXA-scanning. Bone mineral density measurements of the tibiotarsal region were made with a fan beam X-ray densitometer (Model QDR-4500A; Hologic, Waltham, Mass.). Because of the small size of rat bones, an ultra-high-resolution software program (Hologic) was utilized to increase the number of lines scanned by fourfold and to slow the speed of the scanning arm to produce an oversampling; resolution was increased more than sevenfold compared with a typical human scan (manufacturer's data, not shown). For small animals, an additional small collimator that was adjustable between 1–9 mm in diameter was inserted over the original

Table 3. Berenbaum's interaction index for inhibition of loss of body weight.

PEG sTNF-RI (mg/kg)	IL-1ra (mg/kg per hour)	Percent inhibition ± SE		Berenbaum's interaction index	
		observed	predicted		
0.00	0.0	0.0 ± 5.0	3.3 ± 4.5		
0.00	0.2	5.7 ± 4.9	7.3 ± 4.3	1.00	
0.00	1.0	35.1 ± 8.7	21.5 ± 6.7	1.00	
0.00	5.0	20.9 ± 4.9	21.5 ± 4.9	1.00	
0.25	0.0	11.2 ± 5.3	11.7 ± 4.5	1.00	
1.00	0.0	24.5 ± 3.4	23.4 ± 3.1	1.00	
4.00	0.0	36.2 ± 7.9	37.5 ± 7.7	1.00	
0.25	0.2	31.2 ± 6.4	29.1 ± 5.2	0.55	← synergy
0.25	1.0	29.6 ± 8.3	30.6 ± 8.1	0.12	← synergy
1.00	0.2	48.6 ± 5.6	49.2 ± 5.5	0.03	← synergy
1.00	1.0	43.3 ± 6.6	43.1 ± 6.6	0.29	← synergy
4.00	5.0	100.0 ± 3.0	100.0 ± 3.0	0.55	← synergy

Data from the study shown in figure 1 were modeled according to Carter et al. [54]. The following equation generates predicted values: predicted% inhibition $= 0.197 + 0.058X_1 + 0.065X_2 + 3.882X_1X_2 - 1.591X_1^2X_2 - 2.857X_1X_2^2 + 0.839X_1^2X_2^2 +$ random effects with $X_1 =$ IL-1ra and $X_2 =$ PEG sTNF-RI.

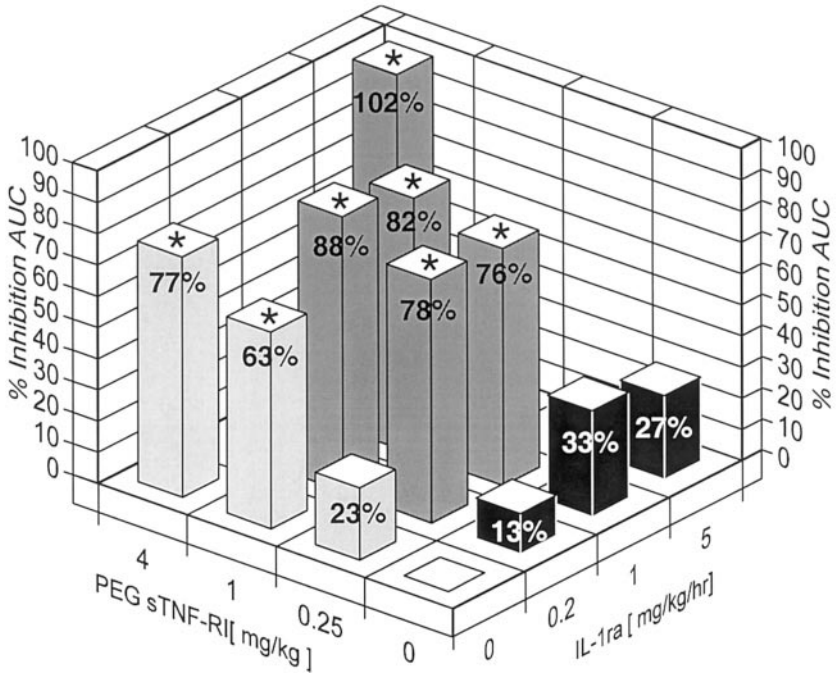

Figure 2. Inhibition of paw swelling by combination treatment with IL-1ra and PEG sTNF-RI. Arthritis was induced and Lewis rats were treated as described in figure 1 and table 1. Paw swelling was used as a measure of inflammation. The volume of hind paws was measured daily as described in Materials and methods. The data shown are the inhibition of paw swelling calculated by the area under the curve (AUC) from day 9 to 16. For clarity, error bars are omitted; for SEs and the results of the isobologram analysis see table 4. *$p < 0.05$ Mann-Whitney test.

collimator to further focus the beam. The dual-beam scheme incorporates X-rays of two different energy levels to achieve differential penetrance of bone, fat and muscle, thereby allowing subtraction of the soft tissue within a selected area and leaving only bone to be scanned and measured. The time required for a typical scan was 1–2 min. During a scan, paws were oriented horizontally relative to the detector. Following scanning, a region of interest was selected for analysis by positioning a rectangular box (29 × 25 mm) centered at the calcaneus. After bone areas were adjusted, proprietary algorithms within the software (Hologic) calcu-

Table 4. Berenbaum's interaction index for inhibition of inflammation.

PEG sTNF-RI (mg/kg)	IL-1ra (mg/kg per hour)	Percent inhibition ± SE		Berenbaum's interaction index	
		observed	predicted		
0.00	0.0	0.0 ± 9.7	5.6 ± 8.8	1.00	
0.00	0.2	13.1 ± 8.8	15.1 ± 8.0	1.00	
0.00	1.0	33.4 ± 12.8	30.2 ± 10.5	1.00	
0.00	5.0	26.5 ± 13.7	27.0 ± 13.6	1.00	
0.25	0.0	22.6 ± 5.3	23.0 ± 5.1	1.00	
1.00	0.0	63.2 ± 10.6	56.0 ± 9.4	1.00	
4.00	0.0	77.1 ± 6.5	77.7 ± 6.5	1.00	
0.25	0.2	77.6 ± 9.9	71.6 ± 8.9	0.51	← synergy[a]
0.25	1.0	75.7 ± 5.3	76.1 ± 5.3	0.00	← synergy[b]
1.00	0.2	87.7 ± 5.1	88.2 ± 5.1	0.14	← synergy[c]
1.00	1.0	82.2 ± 3.1	82.2 ± 3.1	0.29	← synergy[d]
4.00	5.0	102.1 ± 1.5	102.1 ± 1.5	0.81	

Data from the study shown in figure 2 were modeled according to Carter et al. [54]. The following equation generates predicted values: predicted% inhibition $= 0.229 + 0.147X_1 + 0.009X_2 + 7.16X_1X_2 - 3.769X_1^2X_2 - 4.483X_1X_2^2 + 1.517X_1^2X_2^2 +$ random effects with $X_1 =$ IL-1ra and $X_2 =$ PEG sTNF-RI.
a–d are shown in figure 8 together with the calculated 80% isobole.

lated bone area, bone mineral content, and bone mineral density.

Histopathology. After DEXA analysis, ethanol-fixed hindpaws were decalcified by immersion in eight serial changes of a 1:1 mixture of 8N formic acid (VWR) and sodium formate (VWR). Subsequently, paws were divided longitudinally along the median axis, dehydrated, and embedded in paraffin. For each ankle, a 4-µm-thick sagittal section was stained with hematoxylin and eosin (HE). Three components of the arthritic process in the distal tibia and tarsal bones as well as the surrounding fascia were assessed using a 'blinded' analytical paradigm. These endpoints included inflammation (in peri-articular soft tissues and bone marrow), skeletal remodeling (bony degeneration and repair, as well as destruction of cartilage), and bone erosion (bone destruction only). These lesions were scored separately for each hind paw of every rat using semi-quantitative grading scales (table 2) adapted in part from a published scoring system [24].

Statistical analysis. Statistical significance of differences for clinical and histopathological measures was assessed by analysis of variance based on the Mann-Whitney U test using commercial software (Statsoft, Tulsa, Okla.). The isobologram method [52, 53] was applied to differentiate between additive versus synergistic efficacy for the various combinations of IL-1ra and PEG sTNF-RI, with the data modeled according to Carter and coworkers [54]. Synergy is obtained if the effect of the drug combination requires less of each drug to achieve the same effect as each drug alone [55]. A dose-response surface model (RSM) was used to analyze the data as follows:

$$y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{12}X_1X_2 + \beta_{112}X_1^2X_2 + \beta_{122}X_1X_2^2 + \beta_{1122}X_1^2X_2^2 + \text{random effects} \quad (1)$$

where y is the response or dependent variable, e.g., percent inhibition. The βs are the regression coefficients for the exploratory or independent variables e.g., IL-1ra (X_1) and PEG sTNF-RI (X_2). In model 1, the random effects represent variability in the levels of the treatments alone and in combination. They were added to improve the predictive ability of the RSM. The estimates of slope, βs, were subsequently used to compute Berenbaum's interaction index according to the formula:

$$B_{index} = 1 - (\beta_{12}X_1X_2 + \beta_{112}X_1^2X_2 + \beta_{122}X_1X_2^2 + \beta_{1122}X_1^2X_2^2)/(Y - \beta_0) \quad (2)$$

where Y is the predicted response at the particular combination of the experimental design. To determine if a particular combination deviates far from the line of additivity, specific tests were performed to simultaneously evaluate whether the interaction coefficients β_{12}, β_{112}, β_{122}, and β_{1122} are zero using SAS on the UNIX platform [56]. All results were expressed as the average ± SE.

Results

Weight loss and mobility. Adjuvant arthritis is a systemic disease in rats, and marked weight loss is one of the major clinical sequelae (fig. 1). Treatment with either IL-1ra or PEG sTNF-RI alone prevented part but not all of the weight loss. However, combined therapy with both IL-1ra and PEG sTNF-RI, at all combina-

tions of these two agents, resulted in stronger inhibition of weight loss than either IL-1ra or PEG sTNF-RI alone. The co-administration of IL-1ra at 5 mg/kg per hour and PEG sTNF-RI at 4 mg/kg per day (group 13) completely prevented weight loss. In fact, the growth curves of the rats in this group were indistinguishable from normal, non-arthritic rats (fig. 1). Furthermore, the rats in this group behaved and moved like normal rats and, in fact, could not be distinguished from normal rats by observers blinded to the treatments (data not shown). Statistical analysis of the data using the isobologram method demonstrated synergy for the inhibition of weight loss for all combinations of IL-1ra with PEG sTNF-RI (table 3).

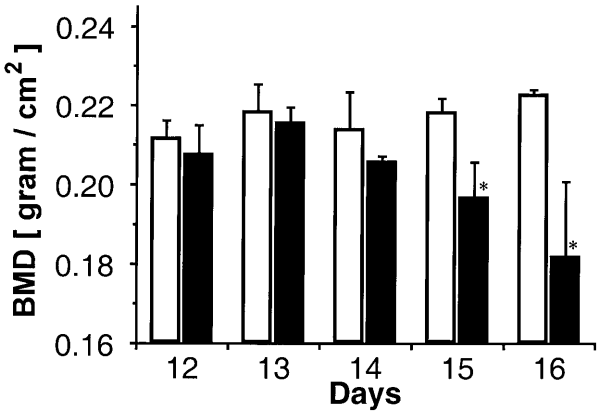

Figure 3. Marked loss of bone mineral density (BMD) in bones of the tibiotarsal joint during the course of adjuvant arthritis. Arthritis was induced in Lewis rats by injection of heat-killed mycobacteria in oil as described in Materials and methods. Groups of normal (open bars) and arthritic (filled bars) rats were sacrificed on the days indicated. Bone mineral density was measured in the tibiotarsal region as described in Materials and methods. In mycobacteria-induced adjuvant arthritis, severe inflammation and erosion occurs in this region accompanied by marked loss in bone mineral density in remaining bone structures. This loss of bone mineral densisty becomes apparent at day 14 and is statistically significant by day 15, i.e., 5 days following clinical onset of arthritis. *$p < 0.05$ Mann-Whitney test.

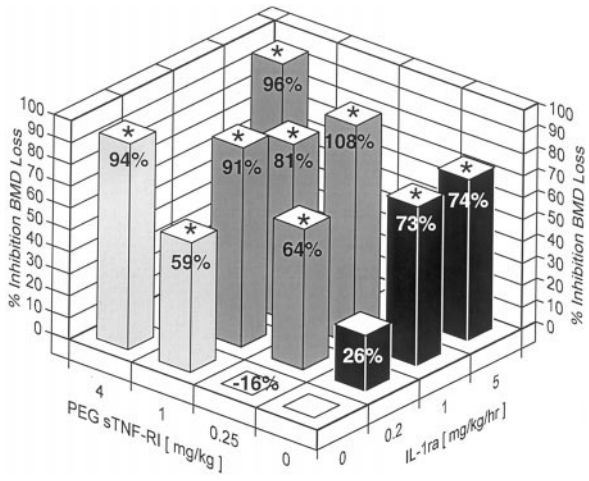

Figure 4. Inhibition of loss of bone mineral density by combination treatment with IL-1ra and PEG sTNF-RI. Arthritis was induced and Lewis rats were treated as described in figure 1 and table 1. On day 16, bone mineral density was measured by DEXA in the tibiotarsal region as described in Materials and methods. Bars represent the percent inhibition of the loss of BMD relative to that of untreated arthritis control (Group 2, table 1). For clarity of the figure, error bars are omitted; for SEs and the results of the isobologram analysis see, table 5. *$p < 0.05$ Mann-Whitney test.

Inflammation. The extent of inflammation was quantified clinically using hindpaw volume measurements as an index of paw swelling (fig. 2). The maximum inhibition achieved with IL-1ra alone was typically about 30%. With PEG sTNF-RI, a dose-dependent inhibition of paw swelling of up to 77% was observed. Combination treatment with IL-1ra and PEG sTNF-RI resulted in much greater reduction in the degree of inflammation. For example, IL-1ra at 0.2 mg/kg per hour resulted in an inhibition of $13 \pm 9\%$ and PEG sTNF-RI at 0.25 mg/kg in $23 \pm 5\%$, whereas combination treatment with IL-1ra and PEG sTNF-RI at these low doses resulted in $78 \pm 10\%$ inhibition of paw swelling. The isobologram analysis for inhibition of inflammation revealed that most combinations of IL-1ra with PEG sTNF-RI exhibited synergistic activity (table 4).

Bone mineral density. In arthritic rats, bone mineral density has been measured using DEXA in, e.g., vertebrae, femur, tibia, and the tibiotarsal region [44, 45, 48–51]. Since bony lesions are most prominent in hind paws of rats with adjuvant arthritis, we investigated the calcaneus as a suitable rapid means for quantifying disease-induced reductions in bone mineral density [57]. Loss of bone mineral density at this site gradually develops during the first week following onset of clinical arthritis and becomes significant by day 15 after mycobacterial inoculation (fig. 3). Therefore, for the pharmacological studies of efficacy presented here, day 16 was chosen to measure bone mineral density. Treatment with IL-1ra or PEG sTNF-RI alone resulted in a dose-dependent prevention of bone mineral density loss of up to 74 ± 15 or $94 \pm 15\%$, respectively (fig. 4). As with paw swelling, treatment with combinations of IL-1ra and PEG sTNF-RI was more efficacious than treatment with the individual drugs alone (fig. 4). For example, IL-1ra at 0.2 mg/kg per hour resulted in an inhibition of $26 \pm 23\%$ and PEG sTNF-RI at 0.25 mg/kg in $-16 \pm 22\%$. In contrast, combination treatment with IL-1ra and PEG sTNF-RI at these low doses resulted in $64 \pm 14\%$ inhibition of loss of bone mineral density. The isobologram analysis demonstrated that various combinations of IL-1ra and PEG sTNF-RI acted synergistically to inhibit loss of bone mineral density (table 5).

Table 5. Berenbaum's interaction index for inhibition of loss of bone mineral density.

PEG sTNF-RI (mg/kg)	IL-1ra (mg/kg per hour)	Percent inhibition ± SE		Berenbaum's interaction index	
		observed	predicted		
0.00	0.0	0.0 ± 25.9	13.6 ± 19.1	1.00	
0.00	0.2	26.0 ± 22.5	26.8 ± 17.3	1.00	
0.00	1.0	72.7 ± 9.4	67.7 ± 9.0	1.00	
0.00	5.0	74.4 ± 15.3	76.9 ± 15.2	1.00	
0.25	0.0	−16.1 ± 22.3	6.8 ± 18.1	1.00	
1.00	0.0	59.2 ± 15.7	54.4 ± 13.5	1.00	
4.00	0.0	93.9 ± 15.1	94.4 ± 15.0	1.00	
0.25	0.2	63.6 ± 13.8	62.9 ± 12.1	0.18	← synergy
0.25	1.0	107.6 ± 19.6	107.9 ± 19.3	0.18	← synergy
1.00	0.2	90.5 ± 9.6	90.7 ± 9.5	0.30	← synergy
1.00	1.0	80.8 ± 8.1	80.7 ± 8.1	0.52	← synergy
4.00	5.0	95.8 ± 9.4	95.8 ± 9.4	1.76	

Data from the study shown in figure 4 were modeled according to Carter et al. [54]. The following equation generates predicted values: predicted% inhibition $= 0.320 + 0.156X_1 + 0.099X_2 + 6.956\ X_1X_2 - 4.459X_1^2X_2 - 3.744X_1X_2^2 + 1.479X_1^2X_2^2 +$ random effects with $X_1 =$ IL-1ra and $X_2 =$ PEG sTNF-RI.

Histopathology. While pathology findings varied to a modest degree between animals within a group as well as between right and left ankles for a rat, lesions exhibited a clearly defined pattern encompassing both destructive and reparative processes (fig. 5). Dense accumulations of mixed inflammatory cells and numerous osteoclasts were present in association with erosions of cortical and trabecular regions of the tarsal bones and tibia. Production of new bony trabeculae along periosteal and endosteal surfaces was extensive. Joint cartilages were often isolated by erosion of the subjacent epiphyseal bone, but remained intact. Neutrophils, newly formed capillaries (i.e., angiogenesis), hyperplastic stromal cells, and sometimes edema were the most prominent inflammatory changes in the peri-articular soft tissues.

The severity of inflammation generally was marked (grade 4) in rats with untreated arthritis (group 2, table 1). Marked inflammation also occurred in all groups given any dose of either IL-1ra or PEG sTNF-RI alone; therapy with a high dose of IL-1ra (5 mg/kg per hour) or PEG sTNF-RI (4 mg/kg) resulted in non-significant reductions in inflammation severity of 17 ± 5% and 21 ± 8%, respectively. In contrast, average inflammation scores were reduced significantly in most groups given both IL-1ra and PEG sTNF-RI (figs 5, 6). For example, simultaneous therapy with IL-1ra at 5 mg/kg per hour and PEG sTNF-RI at 4 mg/kg (group 13, table 1) resulted in significant inhibition of 67 ± 5%, yielding a grade statistically similar to that defined for the non-arthritic control animals (group 1, table 1). Even the combination of the lowest IL-1ra (0.2 mg/kg per day) and PEG sTNF-RI (0.25 mg/kg) doses (group 9, table 1) provided a significant reduction of 25 ± 9%. By comparison, neither agent affected the severity of inflammation when administered singly at these low doses.

The severity of skeletal remodeling (representing a composite grade for both destructive and reparative bony responses) was generally marked (commonly grade 5) in rats of the untreated arthritis control cohort (group 2, table 1). Marked skeletal remodeling also occurred in all groups given any dose of either IL-1ra or PEG sTNF-RI alone except for the high PEG sTNF-RI dose (4.0 mg/kg; group 5, table 1), which significantly inhibited skeletal changes by 38 ± 13%. In contrast, average skeletal remodeling scores were reduced significantly in most groups given both IL-1ra and PEG sTNF-RI (figs 5, 7); improvement to a comparable degree was noted for bone erosion scores (data not shown). For example, simultaneous therapy with high doses of IL-1ra (5 mg/kg per hr) and PEG sTNF-RI (4 mg/kg) resulted in significant inhibition of 82 ± 9% (group 13, table 1), a grade statistically similar to that observed in the non-arthritic control animals (group 1, table 1). Concurrent therapy with the lowest doses of IL-1ra (0.2 mg/kg per day) and PEG sTNF-RI (0.25 mg/kg) yielded a significant reduction of 42 ± 11% (group 9, table 1). When administered singly at these low doses, neither agent affected the severity of bone damage.

Isobologram analysis. Isobologram analysis defines the complexity of the interaction between two drugs. For simple interactions, synergy is suggested when the interaction index is lower than 1, i.e., below the line of additivity. For antagonism, the index is greater than 1, i.e., above the line of additivity [52, 53]. For the isobologram analysis, data were modeled according to an RSM [54].

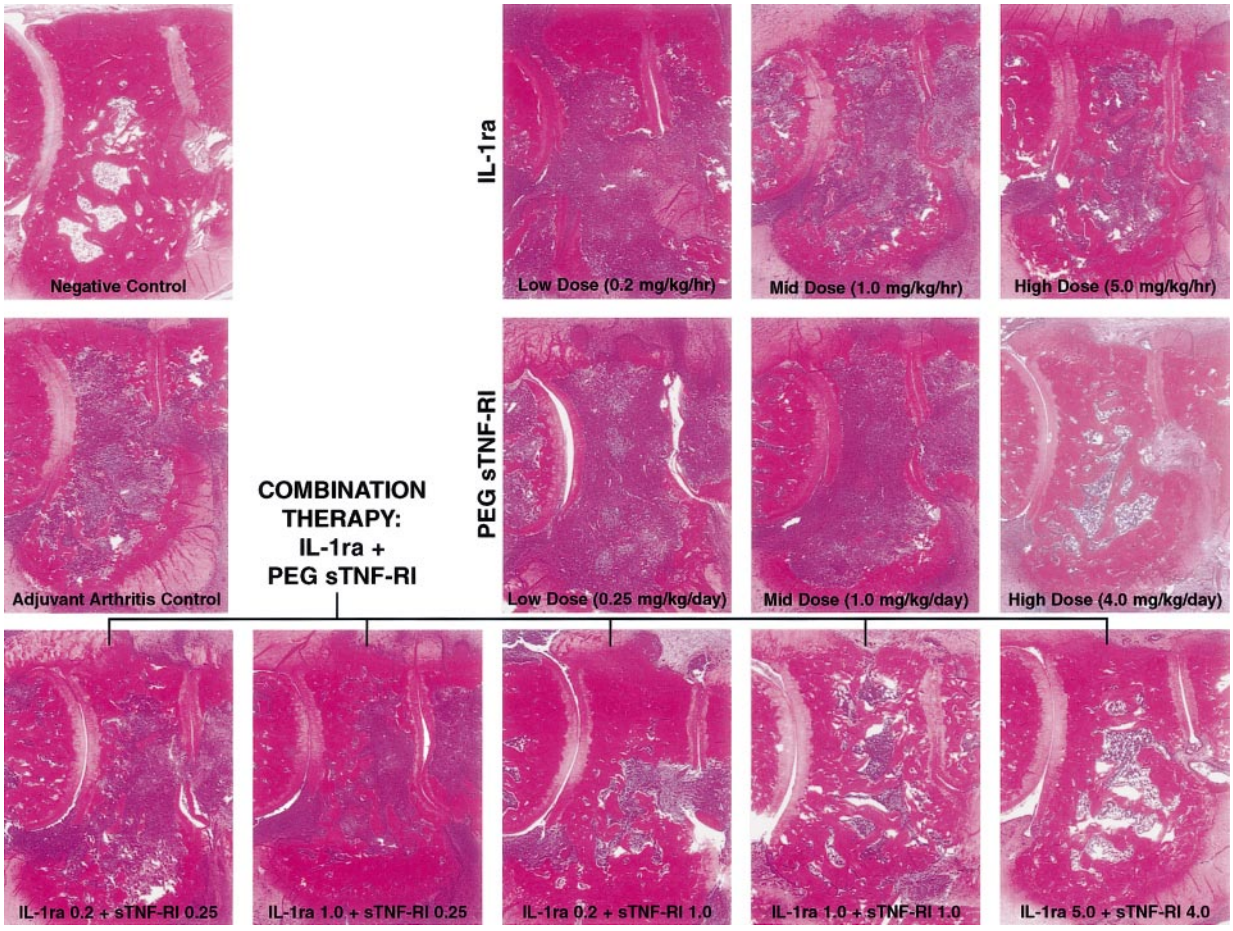

Figure 5. Prevention of inflammation and bone destruction by combination treatment with IL-1ra and PEG sTNF-RI. Arthritis was induced and Lewis rats were treated as described in figure 1 and table 1. On day 16, hindpaws of rats were processed for histopathology as described in Materials and methods. In hindpaws of normal rats, principal features include dense bony cortices, intact articular cartilages, and fat-filled marrow cavities containing scattered hematopoietic precursor cells. Bone and joint structure are severely disrupted in the arthritis control. Major characteristics are severe mononuclear infiltration in the bone marrow and pannus, and advanced destruction of cortical, subchondral, and trabecular bone. Continuous infusion with escalating doses of IL-1ra or daily subcutaneous injection with escalating doses of PEG sTNF-RI on days 9–16 partially prevented bone destruction and inflammation at the highest doses. Treatment with various dose combinations of IL-1ra and PEG sTNF-RI on days 9–16 greatly inhibited inflammation and skeletal damage. Efficacy was observed when low IL-1ra and PEG sTNF-RI doses (ineffective as single-agent therapy) were combined, while high-dose combination completely prevented destruction. H&E staining. Magnification $\times$ 50.

Tables 3–7 show the results of this analysis applied to the data for inhibition of loss of body weight, inflammation, and loss of bone mineral density, as well as for histopathology inhibition of inflammation and skeletal remodeling. The Berenbaum interaction indices clearly indicate a high degree of synergy for most of the combinations tested. Only for high doses, where the effect of single-agent anti-cytokine treatment had already resulted in substantial inhibition, was synergy lacking in some parameters; nevertheless, the effect on body weight or histopathology was synergistic even at the high-dose combination (tables 3, 6, 7, figs 1, 6, 7). This apparent discrepancy was explained simply by the fact that inhibition is limited to 100%, and that for paw swelling and loss of bone mineral density, the palliative effect of PEG sTNF-RI at the high dose was already greater than 75% (tables 4, 5, figs 2, 4). Figure 8 shows the 80% isobole for inhibition of inflammation (as calculated from the equation given in table 4) to illustrate graphically the marked degree of synergism induced by combination therapy with IL-1ra and PEG sTNF-RI at the combination of low and mid doses. Isobole graphs showing extensive synergism by IL-1ra and PEG sTNF-RI for the inhibition of loss of body weight, loss of bone mineral density, or histopathology were essentially identical to that for inflammation (not shown).

Discussion

The goal of the study presented here was to analyze quantitatively the effects of IL-1 and TNF-α inhibition in the model of mycobacteria-induced adjuvant arthritis in the Lewis rat. Immunization of Lewis rats with mycobacteria in oil results in a severe arthritis [46] which cannot be inhibited completely by either IL-1 or TNF-α alone when treatment is therapeutic, i.e., starting at onset of clinical symptoms of arthritis or later (Figs 1, 2, 4–7). In contrast, other models of arthritis, such as collagen-induced arthritis in Lewis rats or DBA/1 mice, can be inhibited completely by both IL-1 or TNF-α inhibition alone (data not shown). Mycobacteria-induced adjuvant arthritis in Lewis rats appeared to us to be uniquely suited for a study which addresses whether combination treatment with IL-1ra or PEG sTNF-RI would result in (i) a benefit, or (ii) additive or synergistic effects, or (iii) therapeutic effects exceeding the effects of treatment with each agent alone.

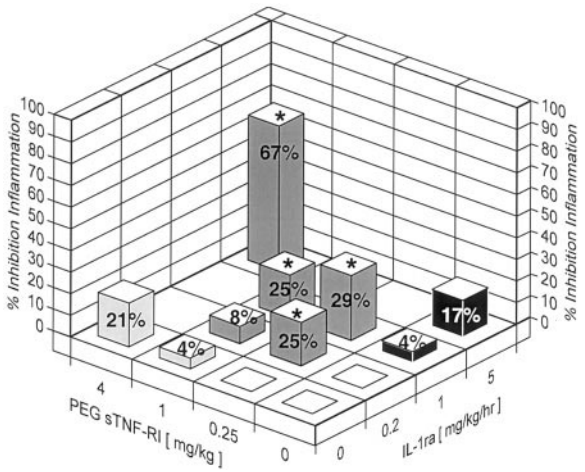

Figure 6. Effect on histopathologic score for paw inflammation by combination treatment with IL-1ra and PEG sTNF-RI. Arthritis was induced and Lewis rats were treated as described in figure 1 and table 1. Hindpaws of rats were processed for histopathology and scored as described in Materials and methods. Bars represent the percent reduction in the mean inflammation score (table 2) relative to that of the untreated arthritis control (Group 2, table 1). *$p < 0.05$ Mann-Whitney test.

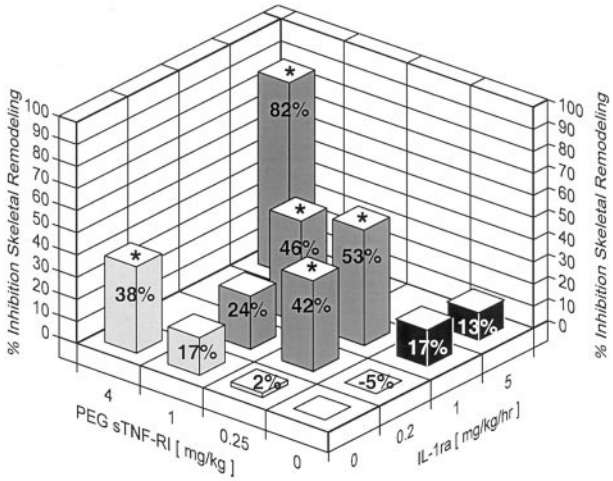

Figure 7. Effect on the histopathologic score for skeletal remodeling (a composite of bony destructive and reparative changes) by combination treatment with IL-1ra and PEG sTNF-RI. Arthritis was induced and Lewis rats were treated as described in figure 1 and table 1. Hindpaws of rats were processed for histopathology and scored as described in Materials and methods. Bars represent the percent reduction in the mean skeletal remodeling score (table 2) relative to that of the untreated arthritis control (Group 2, table 1). *$p < 0.05$ Mann-Whitney test.

As expected, neither IL-1ra nor PEG sTNF-RI alone inhibited the sequelae of mycobacteria-induced adjuvant arthritis completely (Figs 1, 2, 4–7). In contrast, combination therapy with both IL-1ra and PEG sTNF-RI resulted in much greater inhibition of inflammation, loss of body weight, and loss of bone mineral density (Figs 1, 2, 4–7). A very promising aspect of our current data was that low doses of IL-1ra (0.2 mg/kg per hour) and PEG sTNF-RI (0.25 mg/kg per day) which, when given individually, did not significantly impact any parameter of disease, in combination resulted in significant inhibition of both clinical and microscopic measurements relevant to inflammation (76% or greater), skeletal damage (64% or greater), and body weight loss (38% or greater). On the other hand, treatment with the high doses of both IL-1ra (5 mg/kg per hour) and PEG sTNF-RI (4 mg/kg per day) exceeded the maximum effects seen with either treatment alone. In fact, this group of rats did not show any sign of weight loss (fig. 1) and in their behavior they were indistinguishable from normal healthy rats (not shown).

A thorough statistical analysis using the isobologram method [52, 53] indicated that the effects of combination treatment with IL-1ra and PEG sTNF-RI observed in our study in fact were synergistic (tables 3–7, fig. 8). The 80% isobole and the combinations of IL-1ra and PEG sTNF-RI which resulted in $\sim$80% inhibition of inflammation as illustrated in figure 8 highlights the striking reduction in dose(s) of IL-1ra and PEG sTNF-RI possible when both are applied in combination compared to treatment with each drug individually. To our knowledge, our findings provide the first clear evidence that blockade of two pro-inflammatory cytokines will yield synergistic reductions in the clinical and pathologic markers of severe arthritic disease.[2]

[2] Data demonstrating ''greater than additive'' efficacy for combinations of IL-1ra and PEG sTNF-RI in therapy of collagen-induced arthritis in rats [58] were presented in abstract form at the same venue in which part of our findings were presented.

Table 6. Berenbaum's interaction index for inhibition of histopathology inflammation score.

PEG sTNF-RI (mg/kg)	IL-1ra (mg/kg per hour)	Percent inhibition ± SE		Berenbaum's interaction index	
		observed	predicted		
0.00	0.0	0.0 ± 0.0	0.3 ± 2.0	1.00	
0.00	0.2	0.0 ± 0.0	1.0 ± 1.9	1.00	
0.00	1.0	4.2 ± 4.2	3.6 ± 1.6	1.00	
0.00	5.0	16.7 ± 5.3	16.7 ± 3.2	1.00	
0.25	0.0	0.0 ± 0.0	1.6 ± 1.8	1.00	
1.00	0.0	4.2 ± 4.2	5.3 ± 2.0	1.00	
4.00	0.0	20.8 ± 7.7	20.1 ± 6.3	1.00	
0.25	0.2	25.0 ± 9.1	10.5 ± 2.5	0.19	← synergy
0.25	1.0	8.3 ± 8.3	11.8 ± 7.4	0.39	← synergy
1.00	0.2	29.2 ± 7.7	31.1 ± 4.8	0.18	← synergy
1.00	1.0	25.0 ± 0.0	24.5 ± 4.9	0.34	← synergy
4.00	5.0	66.7 ± 5.3	66.7 ± 6.6	0.55	← synergy

Data from the study shown in figure 6 were modeled according to Carter et al. [54]. The following equation generates predicted values: predicted% inhibition $= 0.337 + 3.2771X_1 + 4.950X_2 + 217.06X_1X_2 - 185.32X_1^2X_2 - 64.134X_1X_2^2 + 48.379X_1^2X_2^2 +$ random effects with $X_1 =$ IL-1ra and $X_2 =$ PEG sTNF-RI.

Table 7. Berenbaum's interaction index for inhibition of histopathology skeletal remodeling score.

PEG sTNF-RI (mg/kg)	IL-1ra (mg/kg per hour)	Percent inhibition ± SE		Berenbaum's interaction index	
		observed	predicted		
0.00	0.0	0.0 ± 5.3	3.3 ± 6.8	1.00	
0.00	0.2	−5.1 ± 3.6	1.3 ± 6.5	1.00	
0.00	1.0	16.7 ± 8.7	11.0 ± 6.3	1.00	
0.00	5.0	13.0 ± 9.7	13.9 ± 8.7	1.00	
0.25	0.0	2.2 ± 4.9	5.2 ± 6.4	1.00	
1.00	0.0	16.7 ± 6.7	14.8 ± 6.3	1.00	
4.00	0.0	38.4 ± 13.1	38.5 ± 8.7	1.00	
0.25	0.2	42.0 ± 10.7	31.3 ± 6.4	0.37	← synergy
0.25	1.0	23.9 ± 10.9	26.5 ± 8.7	0.10	← synergy
1.00	0.2	52.9 ± 8.7	56.2 ± 8.6	0.13	← synergy
1.00	1.0	45.7 ± 9.3	44.9 ± 8.8	0.27	← synergy
4.00	5.0	81.9 ± 8.7	81.9 ± 8.8	0.56	← synergy

Data from the study shown in figure 7 were modeled according to Carter et al. [54]. The following equation generates predicted values: predicted% inhibition $= 5.222 + 1.843X_1 + 8.355X_2 + 429.03X_1X_2 - 336.90X_1^2X_2 - 157.64X_1X_2^2 + 94.386X_1^2X_2^2 +$ random effects with $X_1 =$ IL-1ra and $X_2 =$ PEG sTNF-RI.

The results presented here also reconfirm the importance and dominance of IL-1 and TNF-α as the two main drivers in arthritic responses [1–6]. Clearly, therapy with IL-1 and TNF inhibitors was more efficacious than either agent alone in reducing the severity of arthritis. The fact that the low, individually ineffective IL-1ra and PEG sTNF-RI doses also exhibited synergism strongly suggests that a clinical paradigm could be developed to simultaneously attack two pro-inflammatory mechanisms while markedly reducing the dose of each agent to be delivered. This last trait is highly desirable, since most anti-IL-1 and anti-TNF molecules currently in clinical trials or use are proteins that require frequent subcutaneous injection.

Comparison of our clinical (figs 1, 2, 4) and morphologic (figs 6, 7) findings provide further compelling evidence for the use of anti-IL-1 and anti-TNF combinations in clinical practice. Current therapeutic regimens for severe employ only a single cytokine inhibitor (albeit often in conjunction with other drugs, such as methotrexate) [27]. The present animal data demonstrate that use of either IL-1ra or PEG sTNF-RI alone will yield significant improvement in joint function (reduced swelling and bone mineral density deficits) but relatively minor amelioration of histopathologic lesions (inflammatory cell infiltrates and bone erosions) (figs 5–7). The persistence of the cell population responsible for the onset and maintenance of joint injury implies

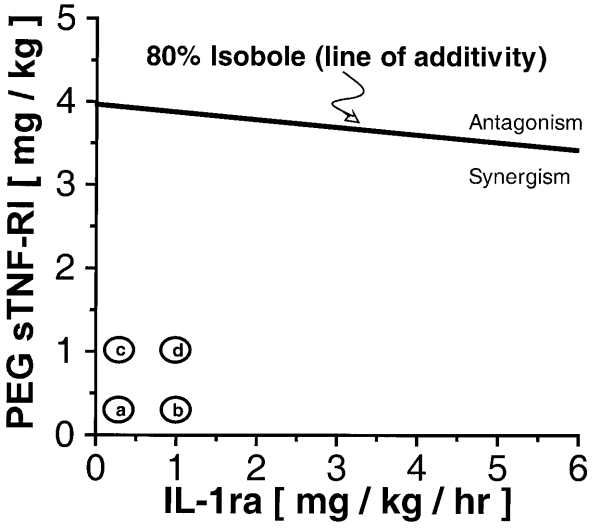

Figure 8. Isobologram for the inhibition of inflammation by combination treatment with IL-1ra and PEGsTNF-RI. The 80% isobole (line of additivity) presented in the figure was calculated based on the equation shown in table 4 and reflects the data shown in figure 2. a–d correspond to the combination treatments as indicated in table 4.

that continued damage would be possible, thereby reducing the degree of efficacy and/or leading to eventual progression of the disease. In contrast, the dual-agent strategy that we tested in the present work clearly shows that concurrent use of two anti-cytokine agents significantly inhibits the microscopic and clinical sequelae in a rat model of severe arthritis. By extrapolation, it seems reasonable to presume that administering anti-IL-1 and anti-TNF in tandem would similarly impact the severity and course of human RA.

IL-1 and TNF-α have been described as the two dominant drivers of arthritic diseases [5, 6]. IL-1 and TNF-α have overlapping but different spectra of biological activities [for reviews see refs 5, 7, 11, 59, 60]. Agents designed to inhibit the actions of IL-1 or TNF-α will obviously function by modifying the signal transduction mechanisms specific to each cytokine, thereby impacting multiple branches of the pro-inflammatory pathways that are responsible for arthritis. However, combination therapy with anti-IL-1 and anti-TNF molecules could also have practical implications with respect to the destructive outcomes of the disease. This possibility is predicated on the hypothesis that regulation of TNF-α activity in arthritis will provide greater inhibition for the inflammatory component of the condition, while control of IL-1 will provide enhanced bone- and cartilage-sparing effects [23, 61–63]. If true, the different functions of IL-1ra and TNF-α in the induction and maintenance of arthritis would support routine co-administration of anti-cytokine combinations as a clinical modality. Our present data suggest that any specificity in the actions of these two cytokines is subtle at best in the adjuvant model of arthritis, with both agents appearing to engender their bone-sparing effects possibly via reduction of intramedullary and soft tissue inflammation (fig. 5). This is also supported by reports that both anti-IL-1 and anti-TNF treatment result in bone-sparing effects in RA [10, 64, 65]. Further work will be required to determine if IL-1ra and PEG sTNF-RI truly impact different clinical and pathologic aspects of RA in the strict sense. However, co-administration of these two agents obviously provides significant synergism in the treatment of inflammatory and bone-destructive events in severe arthritis in animal models. One or both of these anti-cytokines thus seems likely to have considerable future promise in treatment combinations that include other cytokine-inhibiting agents, such as the selective bone-sparing molecule osteoprotegerin [66].

Acknowledgements. The authors wish to thank A. Robin Poole for stimulating discussions and critical comments. The technical support for histology provided by Diane Duryea and Ennis Julian is gratefully acknowledged.

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