Effects of PEGylated soluble tumor necrosis factor receptor type I (PEG sTNF-RI) alone and in combination with methotrexate in adjuvant arthritic rats

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Abstract

Objective

To determine the potential combination benefit of treatment with PEG sTNF-RI and methotrexate in adjuvant arthritic rats.

Methods

Lewis rats with adjuvant arthritis were treated by sc injections of either 3.0 or 0.3 mg/kg PEG sTNF-RI on days 9, 11, and 13 of adjuvant arthritis. The effects of PEG sTNF-RI treatment alone were compared to treatment with daily oral methotrexate (0.075, 0.06 or 0.045 mg/kg) or methotrexate in combination with PEG sTNF-RI. Efficacy was monitored by volume measurement of ankle joints, final paw weights and histologic evaluation with particular emphasis on bone lesions.

Results

Treatment with 3.0 or 0.3 mg/kg PEG sTNF-RI alone resulted in 52% or 28% inhibition, respectively, of paw swelling as assessed by final paw weight. Treatment with methotrexate at either 0.075, 0.06, or 0.045 mg/kg gave 84%, 51% or 18% inhibition and combination treatment resulted in additive inhibitory effects. Histologic evaluation of ankle joints demonstrated 68% or 25% inhibition of bone resorption with PEG sTNF-RI alone at 3.0 or 0.3 mg/kg. Treatment with 0.075, 0.06 or 0.045 mg/kg methotrexate resulted in 98%, 76% or 40% inhibition of bone resorption. Additive benefit was best seen with the lower doses of methotrexate.

Conclusion

Combination therapy with PEG sTNF-RI and methotrexate results in additive benefit, with the final result being excellent inhibition of all arthritis parameters. Data from these studies supports the clinical investigation of the use of combination therapy of PEG sTNF-RI and methotrexate in rheumatoid arthritis patients.

Key words

Tumor necrosis factor, soluble TNF type I receptor, adjuvant arthritis, methotrexate.

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Introduction

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 IL-1 and TNF-α, in the pathogenesis of RA is now well accepted as the result of numerous studies in animal models as well as in patients with the disease (1-5). Soluble TNF receptors and antibodies to TNF have been shown to be clinically efficacious in rheumatoid arthritis patients (6-9). Animal models of arthritis in which these agents were evaluated predicted the excellent human clinical response (10-17).

Methotrexate is a well-accepted therapeutic agent used in the treatment of RA. Its precise mechanism of action is unknown, but effects on the activity of IL-1 and various other proinflammatory molecules have been reported (18, 19).

Rat adjuvant arthritis is an experimental model of polyarthritis which has been widely used for the pre-clinical testing of numerous anti-arthritic agents which are either undergoing pre-clinical or clinical investigation or which are currently being used as therapeutics in this disease. The hallmarks of this model are the reliable onset of robust polyarticular inflammation, marked bone resorption, and periosteal bone proliferation (20-22). Cartilage destruction occurs but is disproportionately mild in comparison to the inflammation and bone destruction that occurs. Low dose methotrexate treatment has been shown to be efficacious in this model (9).

In the present study, we evaluated the efficacy of PEG sTNF-RI alone and in combination with methotrexate (at optimal and suboptimal doses) on inflammation-associated paw swelling and bone resorption in adjuvant arthritic rats (9) in an effort to determine the potential additive or synergistic effects of the combination therapy.

Materials and methods

Animals and materials

Male Lewis rats (250 - 300 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 to a cage in polycarbonate cages and were allowed *ad libitum* access to food and water. All animal use was in accordance with USDA guidelines for humane care.

Recombinant PEG sTNF-RI and its vehicle were produced at Amgen (Boulder, CO). PEG sTNF-RI is a recombinant *E. coli* form of the "high affinity" p55 soluble tumor necrosis Factor Receptor I (sTNF-RI) to which a 30 Kd polyethylene glycol (PEG) molecule is attached (23-25). Methotrexate (prepared in 1% carboxymethylcellulose, dose volume = 5 ml/kg) was purchased from Sigma (St. Louis, MO). Freunds complete (FCA) and was obtained from Sigma. The synthetic adjuvant N,N-dioctylddecyl-N', N-bis(2-hydroxy-ethyl) propanediamine (LA) was obtained from BolderPath Inc. (Nederland, CO).

Induction and evaluation of adjuvant arthritis

Male rats were given single sc injections (at the base of the tail) of 100 µl of FCA to which 5 mg of synthetic adjuvant (LA) was added. Treatments were initiated on day 1 post-adjuvant injection for methotrexate and on day 9 post-injection of adjuvant for PEG sTNF-RI. Arthritis onset occurred on day 9 or day 10 post-adjuvant injection (22).

Volume measurements of the hind paws were done prior to the onset of arthritis, and then every other day until the study was terminated on day 15 post-injection of the adjuvant. 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. Paws were then collected into formalin for histopathologic evaluation. Body weight and spleen weights were also determined.

Ankle joints were collected into 10% neutral buffered formalin for at least 24 hours prior to placement in Surgipath decalcifier I (Grayslake, IL) for approximately one 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. Multiple sections were prepared to insure that

the distal tibia was present with both cortices and that abundant distal tibial medullary space was available for evaluation. Adjuvant arthritic ankles were given scores of 0-5 for bone resorption and inflammation according to the following criteria:

Bone resorption
0: Normal;
1: Minimal, based on small areas of resorption in the distal tibial trabecular or cortical bone, not readily apparent on low magnification, and rare osteoclasts;
2: Mild, defined as more numerous areas of resorption in the distal tibial trabecular or cortical bone, not readily apparent on low magnification, and more numerous osteoclasts;
3: Moderate, reflecting obvious resorption of medullary trabecular and cortical bone without full thickness defects in cortex, loss of some medullary trabeculae, lesions apparent on low magnification, and more numerous osteoclasts;
4: Marked, based on full thickness defects in the cortical bone, often with distortion of the profile of the remaining cortical surface, marked loss of medullary bone of distal tibia, numerous osteoclasts, and no resorption in the smaller tarsal bones;
5: Severe, defined as full thickness defects in the cortical bone, often with distortion of the profile of the remaining cortical surface, marked loss of medullary bone of distal tibia, and numerous osteoclasts, with resorption also present in the smaller tarsal bone.

Inflammation
0: Normal;
1: Minimal infiltration of inflammatory cells in periarticular tissue;
2: Mild infiltration;
3: Moderate infiltration with moderate edema;
4: Marked infiltration with marked edema;
5: Severe infiltration with severe edema.

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

Study design
All test groups except the normal controls (n = 4) contained 7 rats. Treatment with PEG sTNF-RI was by sc injection on days 9, 11 and 13 post-adjuvant injection. Oral methotrexate gavage doses were 0.045, 0.06 or 0.075 mg/kg beginning on day 1 and continuing through day 14. All rats in this study were treated similarly in that all were given oral doses of the vehicle or methotrexate and all were given sc injections of the vehicle or PEG sTNF-RI. Rats were terminated on day 15 for the determinations of final paw, body and spleen weights.

Statistical analysis
Clinical data for paw volume were analyzed by determining the area under the dosing curve with subsequent analysis of variance. For calculation of the AUC, the daily volume of the ankle joints (using a water displacement system) for each rat was entered and plotted using Statistical Analysis Software (SAS, Cary, NC), where the area between the treatment days after the onset of disease to the termination day was computed. The means for each group were determined and the % inhibition from arthritis controls was calculated by comparing values for treated and normal animals. Paw weights and histologic parameters (mean ± SE) for each group were analyzed for differences using the Student's t-test. In both cases, significance was set at $p = 0.05$.
Percent inhibition of paw volume, splenomegaly and AUC was calculated using the following formula: % Inhibition = A - B/A X 100, where A = disease control - normal and B = treated - normal.

Results
Clinical and splenomegaly parameters
Treatment of adjuvant arthritic rats with 3.0 or 0.3 mg/kg PEG sTNF-RI resulted in 53% or 27% inhibition of AUC for paw swelling and 52% or 28% inhibition of the final paw weights (Fig. 1A) Splenomegaly was decreased by 58 and 67% at the 3.0 and 0.3 mg/kg doses. There were no beneficial effects of treatment on body weight (Fig. 1B).
Treatment of adjuvant arthritic rats with daily oral doses of methotrexate (0.075, 0.060 or 0.045 mg/kg) on days 1 to 14 resulted in 87, 65 or 31% inhibition of AUC for paw swelling and 84, 51 or 18% inhibition of the final paw weight (Fig. 2A). Methotrexate treatment inhibited the splenomegaly and body weight effects of adjuvant disease in a dose responsive manner (Fig. 2B).
Concurrent treatment with PEG sTNF-RI and methotrexate generally resulted in additive benefit. This was most obvious at the lower doses of both treatments. Treatment with methotrexate at 0.075 resulted in 80-90% inhibition of clinical parameters. Addition of PEG sTNF-RI resulted in 90-100% inhibition (data not shown). The combination treatment of 3 mg/kg PEG sTNF-RI and 0.06 mg/kg methotrexate resulted in 81% inhibition of AUC for paw swelling and 76% inhibition of final paw weight (Fig. 3A). Combination benefit was not seen on splenomegaly since methotrexate alone at this dose gave 81% inhibition. However, this combination did result in 48% inhibition (significant) of adjuvant disease body weight effects whereas neither treatment alone gave statistically significant effects.
Similar results were observed when rats were given the combination of 3 mg/kg PEG sTNF-RI and 0.045 mg/kg methotrexate (Fig. 4A and B,) in that the best combination benefit was seen on body weight effects. When the combination of 0.3 mg/kg PEG sTNF-R1 and 0.06 mg/kg methotrexate was given, the efficacy on all parameters reflected the efficacy of methotrexate alone (Fig. 5A and B). However, when the two lowest treatment doses were given in combination, 0.3 mg/kg PEG sTNF-RI and 0.045 mg/kg methotrexate, additive benefit was seen on paw swelling parameters, splenomegaly and body weight effects (Fig. 6 and B).

Histologic parameters
Histologic evaluation of ankle joints from rats treated with PEG sTNF-RI at 3 or 0.3 mg/kg demonstrated 53% or 17% inhibition of inflammation and 68% or 25% inhibition of bone resorption, respectively (Fig. 1C). Treatment with methotrexate resulted in dose responsive significant inhibition of these parameters at 0.075, 0.06 and 0.045 mg/kg (Fig. 2C) with the 0.075 mg/kg dose providing

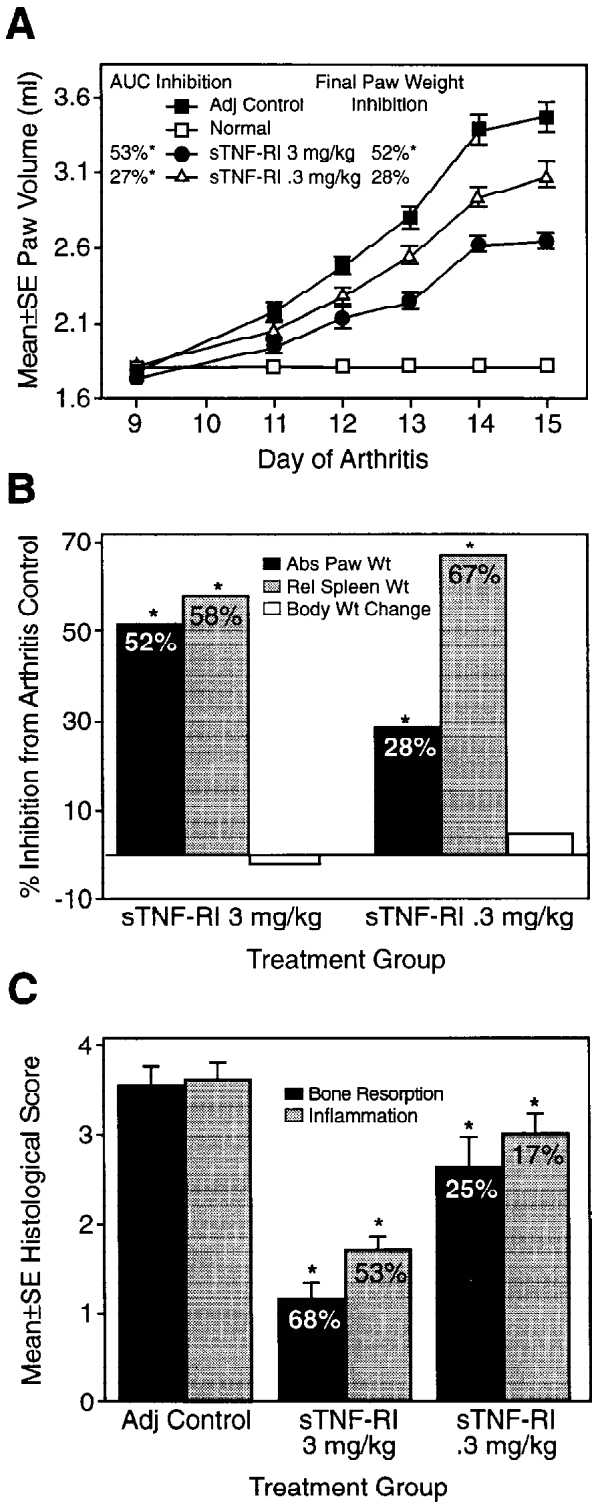

Fig. 1. Paw volume over time (**A**) in adjuvant arthritic rats treated with 3.0 or 0.3 mg/kg PEG sTNF-RI sc, on days 9, 11 and 13 of arthritis. AUC % inhibition and final paw weight inhibition (calculated from raw data) are shown to the left and right of legend. *p≤ 0.05. (**B**) Percent inhibition from control for paw, spleen and body weight calculated from raw data. *p ≤ 0.05. (**C**) Histologic inflammation and bone resorption scores *p ≤ 0.05, 2-tailed T test to adjuvant (**adj**) control, % on bars = % inhibition from adjuvant control.

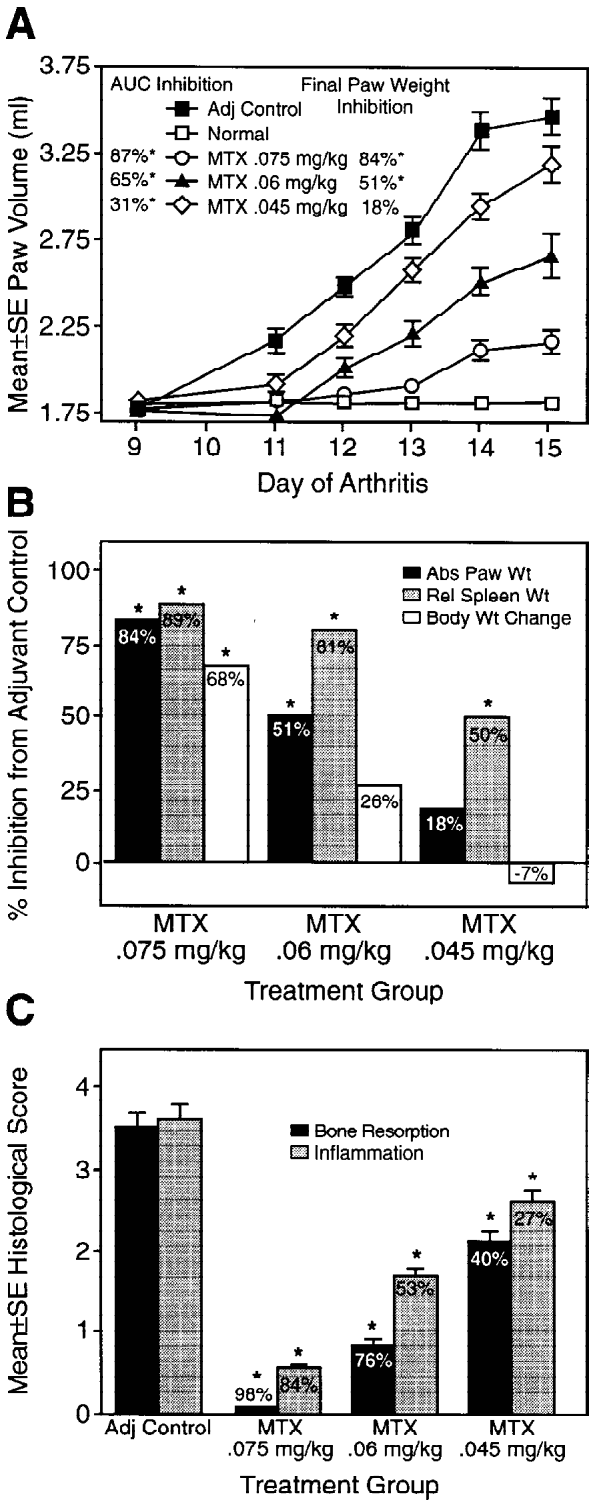

Fig. 2. Paw volume over time (**A**) in adjuvant arthritic rats treated with 0.075, 0.06 or 0.045 mg/kg methotrexate po on days 1-14 of arthritis. AUC % inhibition and final paw weight inhibition (calculated from raw data) are shown to the left and right of legend. *p ≤ 0.05. (**B**) Percent inhibition from control for paw, spleen and body weight calculated from raw data. *p ≤ 0.05. (**C**) Histologic inflammation and bone resorption scores *p ≤ 0.05, 2-tailed T-test to adjuvant (**adj**) control, % on bars = % inhibition from adjuvant control.

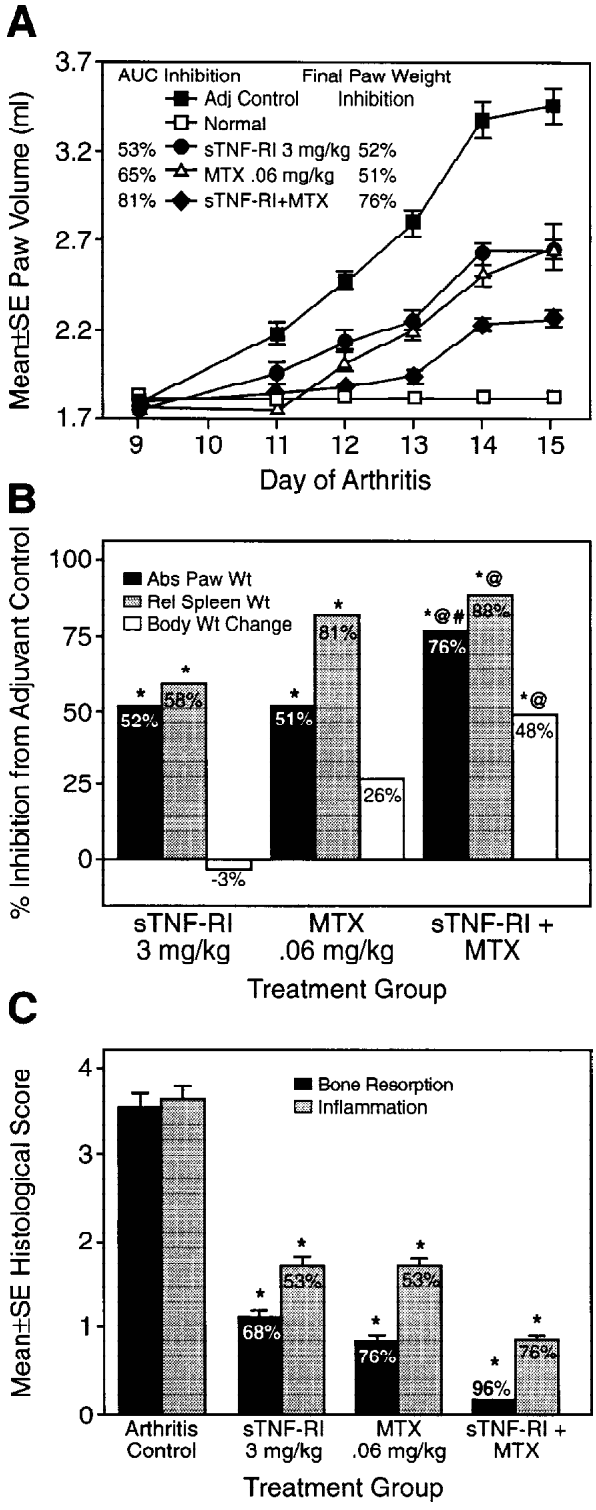

Fig. 3. Paw volume over time (**A**) in adjuvant arthritic rats treated with 0.06 mg/kg methotrexate po on days 1 - 14 of arthritis and 3 mg/kg PEG sTNF-RI sc days 9, 11 and 13 of arthritis. AUC % inhibition and final paw weight inhibition (calculated from raw data) are shown to the left and right of legend. $p \leq 0.05$ comparison to arthritis control, all are significant. (**B**) Percent inhibition from control for paw, spleen and body weight calculated from raw data. *$p \leq 0.05$ comparison to adjuvant control, @$p \leq 0.05$ comparison to sTNF-RI, #$p < 0.05$ comparison to methotrexate. (**C**) Histologic inflammation and bone resorption scores *$p \leq 0.05$, 2-tailed T test to adjuvant (**adj**) control, @sTNF-RI or #methotrexate, % on bars = % inhibition from adjuvant control.

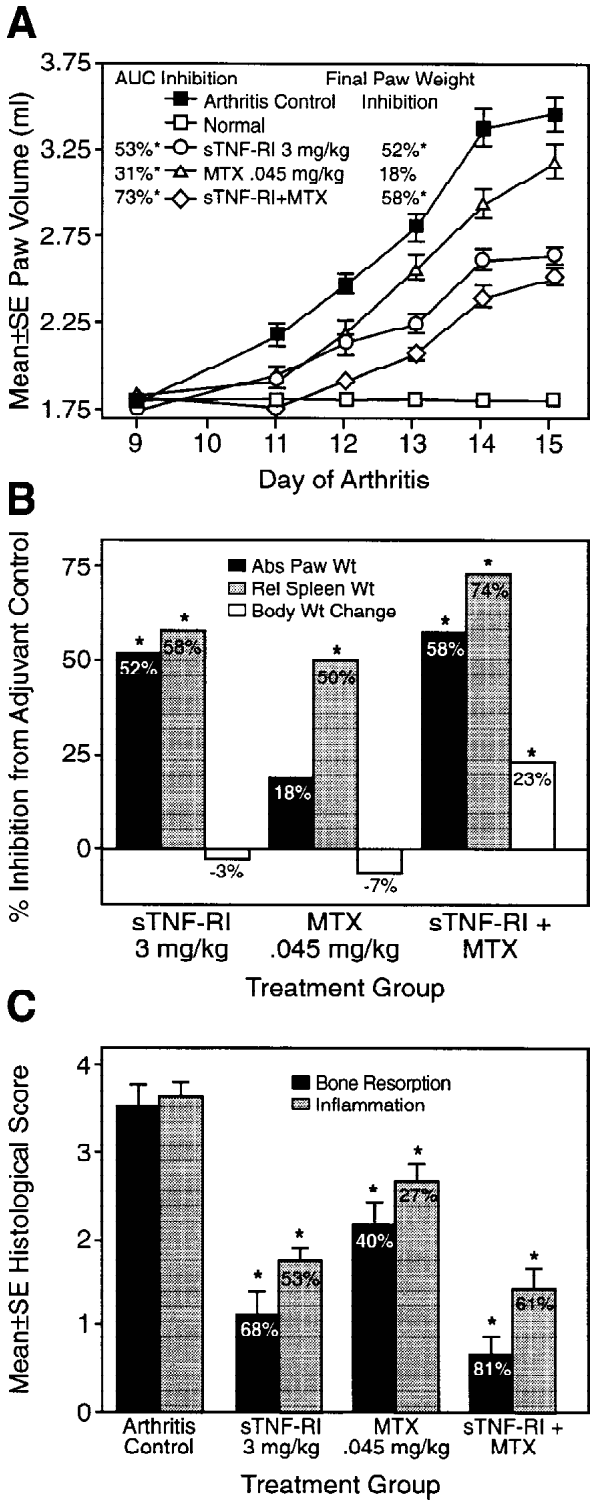

Fig. 4. Paw volume over time (**A**) in adjuvant arthritic rats treated with 0.045 mg/kg methotrexate po on days 1-14 of arthritis and 3 mg/kg PEG sTNF-RI sc days 9,11 and 13 of arthritis. AUC % inhibition and final paw weight inhibition (calculated from raw data) are shown to the left and right of legend. *$p \leq 0.05$. (**B**) Percent inhibition from control for paw, spleen and body weight calculated from raw data. *$p \leq 0.05$ comparison to adjuvant control, @$p \leq 0.05$ comparison to sTNF-RI, #$p<0.05$ comparison to methotrexate. (**C**) Histologic inflammation and bone resorption scores *$p \leq 0.05$, 2 tailed T test to adjuvant (**adj**) control, @sTNF-RI or #methotrexate, % on bars=% inhibition from adjuvant control.

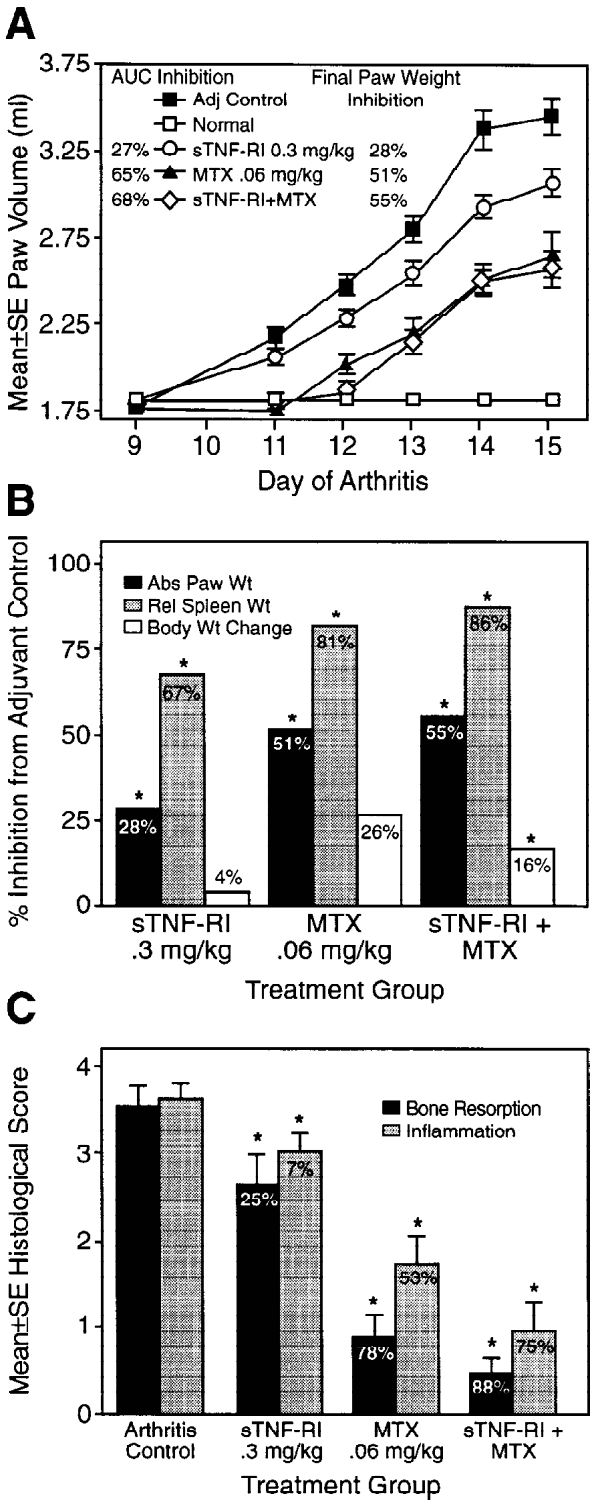

Fig. 5. Paw volume over time (**A**) in adjuvant arthritic rats treated with 0.06 mg/kg methotrexate po on days 1 - 14 of arthritis and 0.3 mg/kg PEG sTNF-RI sc days 9, 11 and 13 of arthritis. AUC % inhibition and final paw weight inhibition (calculated from raw data) are shown to the left and right of legend. $p \leq 0.05$ comparison to arthritis control, all are significant. (**B**) Percent inhibition from control for paw, spleen and body weight calculated from raw data. *$p \leq 0.05$ comparison to adjuvant control, @$p \leq 0.05$ comparison to sTNF-RI, #$p < 0.05$ comparison to methotrexate. (**C**) Histologic inflammation and bone resorption scores *$p \leq 0.05$, 2-tailed T test to adjuvant (**adj**) control, @sTNF-RI or #methotrexate, % on bars = % inhibition from adjuvant control.

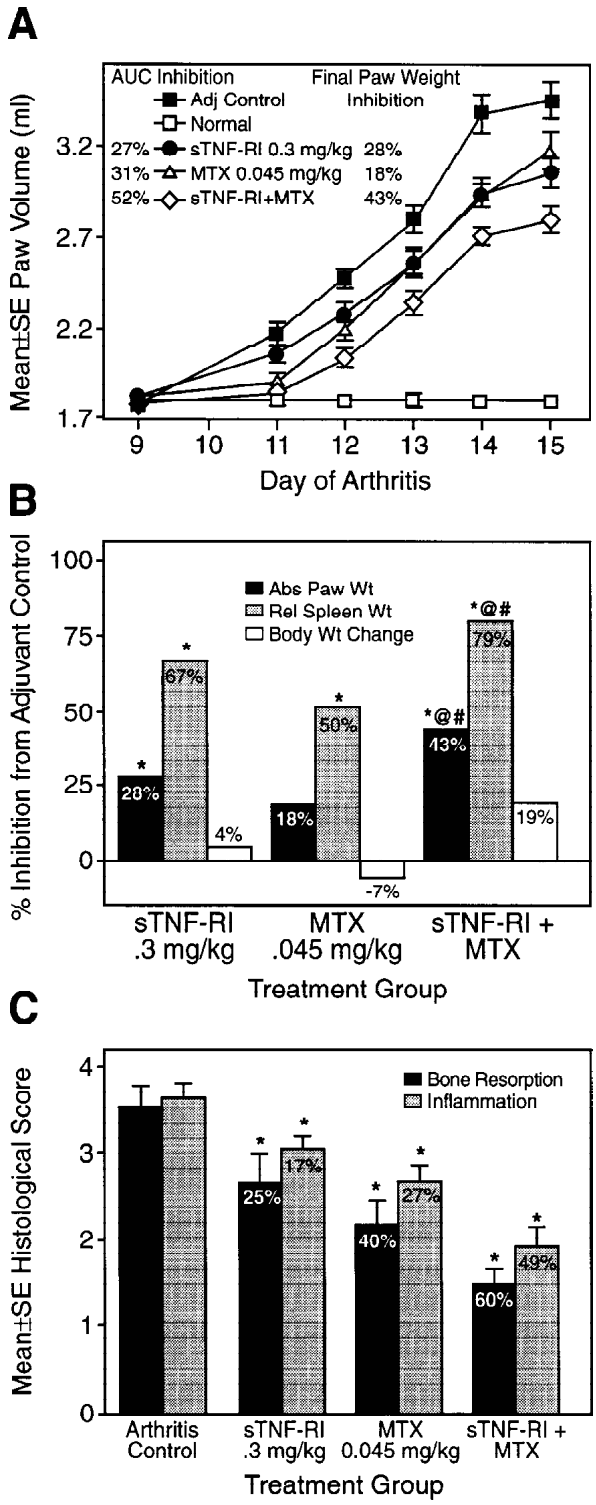

Fig. 6. Paw volume over time (**A**) in adjuvant arthritic rats treated with 0.045 mg/kg methotrexate po on days 1 - 14 of arthritis and 0.3 mg/kg PEG sTNF-RI sc days 9, 11 and 13 of arthritis. AUC % inhibition and final paw weight inhibition (calculated from raw data) are shown to the left and right of legend. $p \leq 0.05$ comparison to arthritis control, all are significant. (**B**) Percent inhibition from control for paw, spleen and body weight calculated from raw data. *$p \leq 0.05$ comparison to adjuvant control, @$p \leq 0.05$ comparison to sTNF-RI, #$p<0.05$ comparison to methotrexate. (**C**) Histologic inflammation and bone resorption scores *$p \leq 0.05$, 2-tailed T test to adjuvant (**adj**) control, @sTNF-RI or #methotrexate, % on bars = % inhibition from adjuvant control.

close to 100% inhibition. Hence, all combinations with 0.075 mg/kg methotrexate were at maximal efficacy (data not shown). The combination of 3 mg/kg PEG sTNF-RI and 0.06 mg/kg methotrexate resulted in 96% inhibition of bone resorption and 76% inhibition of inflammation, both significantly better than either treatment alone (Fig. 3C). The combined effects of 3 mg/kg PEG sTNF-RI and 0.045 mg/kg methotrexate resulted in mildly additive effects on both inflammation and bone resorption (Fig. 4C). Similarly, mildly additive benefit was seen when 0.3 mg/kg PEG sTNF-RI was combined with 0.06 or 0.045 mg/kg methotrexate (Fig. 5C, Fig. 6C).

Discussion

Treatment of adjuvant arthritic rats with PEG sTNF-RI on days 9, 11 and 13 of arthritis resulted in beneficial effects on soft tissue swelling, inflammation and bone resorption. These results suggest that TNF is an important mediator of the inflammation and bone loss in adjuvant arthritic rats.

Low dose methotrexate (0.15 - 0.6 mg/kg/week) has previously been shown to be effective in suppressing paw inflammation in adjuvant arthritic rats (26). Effects on bone resorption were not evaluated in that study. Methotrexate has been shown to inhibit macrophage production of IL-1 in the adjuvant arthritis model (27) and in antigen-induced arthritis in rabbits (28). However, other anti-inflammatory activities of methotrexate (29, 30) may also contribute to the efficacy. In our study, daily treatment with 0.075, 0.06 and 0.045 mg/kg methotrexate provided significant inhibition of clinical arthritis parameters (paw swelling), as well as histologic endpoints of inflammation and bone resorption.

The ED_{50} dose for inhibition of final paw weight was approximately 0.06 mg/kg and the ED_{50} dose for inhibition of bone resorption was 0.05 mg/kg. In other studies, close to 100% suppression of all arthritis parameters occurred with daily methotrexate doses of 0.15 mg/kg (data not shown). However, daily treatment with 0.25 mg/kg of methotrexate results in severe toxicity leading to death (A. Bendele, unpublished observation) after 20-25 days of exposure. Therefore, in this model, a dose (0.25 mg/kg) that is approximately 5 times the ED_{50} (0.05 mg/kg) is lethally toxic. This data suggests that methotrexate has the potential to be extremely effective but that toxicities associated with chronic administration preclude dosing at the extremely efficacious levels.

Therefore an important concept that is advanced with our studies is that lower doses of methotrexate in combination with other agents (preferably nontoxic ones) might provide additive benefit and hence potential for profound disease modification with less risk of deleterious effects. A combination benefit of methotrexate and an anti-TNF antibody (Infliximab) has been described in RA patients (31). Synergistic effects were observed as a result of suppression of anti-globulin responses as well as other undetermined factors. The combination benefit seen in our study was not a result of suppression of the antibody response to protein, as antibodies to sTNF-RI do not occur over the duration of testing that we used (9).

Combination data from our study demonstrates that additive effects occur when PEG sTNF-RI is administered with various doses of methotrexate. The combination benefit was best seen on the bone resorption parameter when the dosage of PEG sTNF-RI was 3 mg/kg and the dosage of methotrexate was 0.06 mg/kg. Combination benefit was most obvious when lower doses (0.06 or 0.045 mg/kg) of methotrexate were used since the higher doses tended to be extremely effective alone.

The toxicities of methotrexate are well defined and definitely limit dosing in the clinic (32). The toxicities of TNF inhibitors are less well defined due to the fact that long-term studies have not been completed with these agents. However, in trials of 3 - 6 months duration, the only consistent adverse effects have been the increased incidence of mild and transient upper respiratory infections (6). The absence of serious adverse events suggests that these agents may have a safety profile that is superior to agents currently used in the treatment of RA. If this benign safety profile is reproduced over the long term and these agents do not show substantial disease progression inhibition on their own, it would seem that combination therapy with agents such as methotrexate might provide excellent beneficial effects to the RA patient.

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