Effects of aging and cytokine blockade on inflammatory cachexia

B. Fathalla[1,2], K. Hamada[1], E. Vannier[1,2], D. Smith[1], C. Edwards III[3], R. Roubenoff[1,2]

[1]Nutrition, Exercise Physiology, and Sarcopenia Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Boston, Massachusetts; [2]Tufts-New England Medical Center, Boston, Massachusetts; [3]Amgen, Inc., Thousand Oaks, California, USA.

Abstract

Objective

To evaluate the role of aging and specific cytokine blockade in the etiology of cachexia caused by adjuvant arthritis (AA), a model of cytokine-associated cachexia.

Methods

AA was induced in Lewis rats using CFA. In Experiment 1, *severity of AA and inflammatory cachexia was assessed in young (Y, age 2-6 months, n = 132) and old rats (O, age 18-22 months, n = 40). In* Experiment 2, *young rats were divided into 5 different intervention groups: Saline-injected (n = 66); CFA-injected (n = 78); CFA-injected and treated with IL-1 receptor antagonist (IL-1Ra, n = 18); CFA-injected and treated with soluble TNF receptor type I (sTNFrI, n = 27); and CFA-injected and treated with both IL-1Ra and sTNFrI (both treatments, n = 8).*

Results

In Experiment 1, *young Lewis rats developed more severe arthritis (mean joint score on day 21 = 5.1 ± 0.3) compared to the old group (0.6 ± 0.6,* $p < 0.0001$*). The young group with AA lost 2.1% of baseline total body weight loss compared to 13.8% total body weight gain in controls (*$p < 0.0001$*). In contrast, old rats injected with CFA lost as much weight (-11%) as age-matched saline injected controls (-13%,* $p > 0.05$*, n = 18, age 18-22 months). In* Experiment 2, *mean joint scores in rats treated with IL-1Ra, sTNFrI or both were higher then untreated rats injected with CFA (*$p < 0.0001$*). Despite this, rats given both IL-1Ra and sTNFrI lost less weight on day 16 (*$p < 0.01$*) and 21 (*$p < 0.002$*) than untreated rats or those rats treated with either IL-1Ra or sTNFrI.*

Conclusion

Lewis rats aged 2-6 months are more susceptible to developing AA than older rats (age range 18-22 months). Inhibition of both IL-1 and TNF is needed to mitigate AA-associated weight loss, and this effect is dissociated from the effect of such inhibition on joint inflammation.

Key words

Adjuvant arthritis, inflammatory cachexia, IL-1 and TNF.

Basil Fathalla, MD, Pediatric Rheumatology Fellow; Koichiro Hamada, DVM; Edouard Vannier, PhD; Donald Smith, PhD; Carl Edwards, III, PhD; Ronenn Roubenoff, MD, MHS, Associate Professor of Medicine and Nutrition, Tufts University.

Supported by a Clinical Science Grant from the Arthritis Foundation, NIH Grants AG15797 and M01-RR00054, USDA Cooperative Agreement 58-1950-9-001, and a grant from Amgen, Inc. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Please address correspondence to: Ronenn Roubenoff, MD, MHS, NEPS Laboratory, JM USDAHNRCA, Tufts University, 711 Washington Street, Boston, MA02111, USA.
E-mail: Roubenoff@mpi.com

Received on June 12, 2003; accepted in revised form on November 11, 2003.

Supported by a Clinical Science Grant from the Arthritis Foundation, NIH Grants AG15797 and M01-RR00054, USDA Cooperative Agreement 58-1950-9-001, and a grant from Amgen, Inc. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Abbreviations:

AA:	Adjuvant arthritis.
CFA:	Complete Freund's adjuvant.
IL-1Ra:	Interleukin-1 receptor antagonist.
PEGsTNFrI:	PEGylated TNF receptor p55.
sTNFrI:	Soluble TNF receptor p55.
TNF:	Tumor Necrosis Factor.

Introduction

Inflammatory cachexia denotes the loss of body cell mass (BCM) that occurs in diseases of chronic inflammation such as rheumatoid arthritis (RA). There is evidence that accelerated protein catabolism in RA correlates positively with an increase in the production of TNF-α and IL-1β in peripheral blood mononuclear cells (PBMC) (1). To further study the mechanisms of inflammatory cachexia, prospective studies in animal models of arthritis and inflammatory cachexia have been used.

Adjuvant arthritis has been found to be a useful model of cytokine-driven inflammatory cachexia (2). In a previous study, we found that young rats injected with CFA had total body weight loss that preceded the development of arthritis by 5 days and continued until day 21 post injection (2). The total body weight loss in rats with AAwas accompanied by a loss in BCM, thus meeting the definition of cachexia. Pair-fed, age-matched controls in the same study lost one-fourth of the weight that AA rats had lost; suggesting that anorexia alone does not explain inflammatory cachexia. There was a strong correlation between TNFα production by unstimulated splenocytes and the amount of weight loss in AA rats. A weaker correlation was observed between IL-1β production in splenocytes cultured with 10 ng/ml LPS and weight loss in the same group. In the present study, we expand on these observations by studying the effects of blockade of one or both of these cytokines in rats susceptible to adjuvant arthritis. Because these studies were carried out over several years, the sample sizes are sufficiently large to give a robust estimate of the effect of age and cytokine inhibition on inflammatory cachexia.

In this study, we compared the severity of arthritis in rats at young and old ages, and assessed the severity of inflammatory cachexia in relation to the severity of arthritis. We also studied the effects of blocking IL-1 and TNFα on inflammatory cachexia in young Lewis rats. In contrast to previous studies, the treatments were administered 3 days after CFA injection, i.e. prior to development of clinical signs of the disease.

Materials and methods

Animals

Male and female Lewis rats bred and maintained in the HNRCA animal facilities were individually housed in 7 x 10 x 7 inch stainless steel suspended rodent cages and provided with the AIN 93 G diet (Teklad, Madison, WI) and water ad libitum for a one week period. Thereafter, rats were sorted by weight and age into experimental groups. The animals were maintained in Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities in an environmentally controlled atmosphere (23ºC, 45% relative humidity, 15 air changes of 100% fresh hepa-filtered air per hour and a 12/12-hour light/dark cycle). Animals were observed daily for clinical signs of distress or disease. Diet weighbacks were performed twice weekly to determine average daily intake. The HNRCAAnimal Care and Use Committee approved the experiment protocol.

Two separate experiments were designed and conducted, as described here below.

Design of Experiment 1

A total of 172 Lewis rats were divided into two groups according to age: Group Y for young rats (age 2-6 months, n = 132) and Group O for older rats (age 18-22 months, n= 40). This experimental design was repeated several times to confirm the results: the total number of animals is shown. After a one-week acclimatization period, rats were injected at the base of the tail with Complete Freund's Adjuvant (CFA; 200 µl of pulverized *Mycobacterium bovis*; Difco, Detroit, MI) in sterile mineral oil (Fisher Scientific, Pittsburgh, PA). Rats were weighed and examined for evidence of joint swelling twice a week. On day 21 post-injection, joint scores were used to grade the severity and extent of arthritis according to the following scale for each limb: 0 = no swelling and full range of motion, 1 = minimal swelling and full range of motion, 2 = moderate/severe swelling and limited range of motion, 3 = moderate/severe swelling and no range of motion. Thus, the maximum score was 12 when

all four limbs had a maximal score of 3. To study the relationship between severity of AA and total body weight loss, we compared mean percent change in body weight from day 0 to day 21 in young rats (data available for 103 rats) with AA from the above Group Y (the control group consists of 50 young rats aged 2-6 months injected with saline) and old rats injected with CFA (data available for 17 rats) from the above Group O (the control group consists of 18 old rats aged 18-22 months injected with saline).

Design of Experiment 2

After a one week acclimatization period as mentioned above, a total of 198 Lewis rats, with an age range of 3 – 5 months, were sorted according to weight and assigned at random to one of five experimental groups:

Group S: Saline-injected (n = 66, 33 males and 33 females).
Group A: CFA-injected (n = 78, 33 males and 45 females).
Group I: CFA-injected, IL-1Ra (n = 18, 9 males and 9 females).
Group T: CFA-injected, sTNFrI (n = 27, 13 males and 14 females).
Group IT: CFA-injected, IL-1Ra and sTNFrI (n = 8, 4 males and 4 females).

The sample sizes for groups S and A are based on pooled experience from several trials. The results of these trials were pooled because they were virtually identical over a two-year period, allowing the results to be combined in order to maximize the statistical power of the study.

Administration of cytokine inhibitors: IL-1 receptor antagonist (Amgen, Inc., Boulder, CO) was administered for 14 days by subcutaneous continuous infusion (2 mg/kg/d) via implantation of Alzet pump 3 days after adjuvant injection. PEG-sTNFrI (Amgen, Inc.) was administered intraperitoneally (3 mg/kg) every 3 days starting 1 day before adjuvant injection.

Statistical analysis

Data were examined graphically and statistically for normality. Results are expressed as mean ± SEM unless otherwise noted. The effect of cytokine blockade and age on body weight change and joint scores was analyzed for statistical differences by ANOVA. Correlations between change in weight and joint score were tested using the Pearson product moment correlation. A two-tailed p-value < 0.05 was considered statistically significant.

Results

Effect of age on severity of adjuvant arthritis

As depicted in Figure 1, the mean joint score on day 21 was 5.1 ± 0.3 out of a possible 12 points for the rats in Group Y (age 2-6 months, n = 132) compared to 0.6 ± 0.6 for the rats in Group O (age 18-22 months, n = 40, $p < 0.0001$).

Relationship between weight loss and adjuvant arthritis

Total body weight records were available for 103 rats from group Y on days 0 and 21. The mean percent change in total body weight from days 0 to 21 was compared to a control group of young rats (age 2-6 months, n = 50, injected with saline). As seen in Figure 2, young rats with AA had a mean weight loss of 2.1% compared to weight gain of 13.8% in the control group ($p < 0.0001$).

Similarly, daily body weights were recorded in the above Group O (data available in 17 rats). The mean percent of change in total body weight from day 0 to day 21 was compared to a control group of old rats (age 18-22 months, n = 18, injected with saline). The old rats injected with CFA lost 11% of their baseline body weight, compared to 13% weight loss in the old rats in the saline-injected group ($p > 0.05$).

The effect of blocking IL-1 and/or TNF on inflammatory cachexia and joint swelling

In Experiment 2, the change in total body weight was compared among five groups of rats on days 4, 8, 12, 16 and 21 as shown in Figure 3. The control group (Group S, injected with saline) showed a mean weight gain of 4.7% by day 21. The Adjuvant Arthritis group with no intervention (Group A) and the IL-1Ra treated group (Group I) had the greatest weight loss, with 4.2% lost by day 21 in each group. The group treated with PEG-sTNFrI (Group T) lost 2.3% of total body weight by day 21 ($p > 0.05$ vs. other groups). In contrast, when weight change between days 0 and 21 was analyzed in the group treated with both IL-1Ra and PEG-sTNFrI (Group IT, Fig. 3) there was a 0.8% increase in weight ($p < 0.002$ vs. controls).

Mean joint scores were paradoxically worse in all three groups that received cytokine blockade (Fig. 4). Mean joint scores of young rats injected with CFA

Fig. 1. Severity of AAin Lewis rats in relation to age. Mean joint scores on day 21 post CFA injection in two groups of rats. GroupY is the young group (age 2-6 months, n = 132) developed severe AAwith a mean joint score of 5.1 ± 0.3. Group O is the old group (age 18-22 months, n = 40) did not develop severe arthritis and had a mean joint score of 0.6 ± 0.6 P< 0.0001 between both groups. * P< 0.0001 (old versus young).

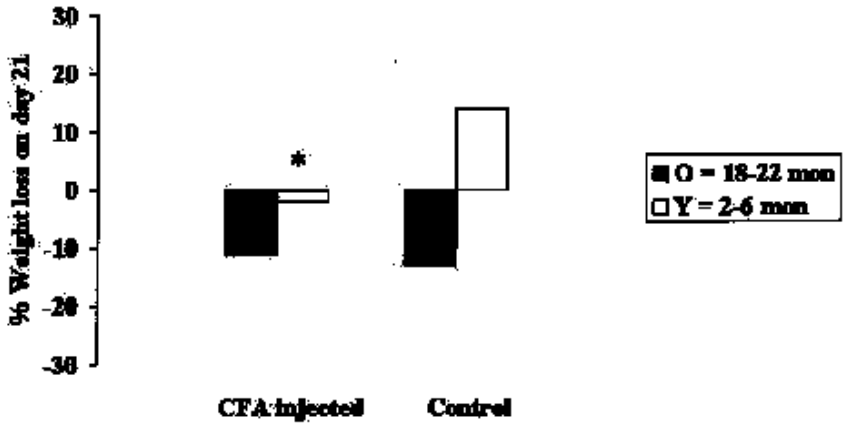

Fig. 2. Severity of weight loss in relation to AA. Mean percent of change in total body weight from day 0 to day 21 post CFA injection was compared between young and old rats. The Young group (rats with AA, age 2-6 months, n = 103) had a mean percent of total body weight loss of 2.1% compared to mean percent of body weight gain of 13.8% in the control group (saline injected, age 2-6 months, n = 50), P< 0.0001.
The Old group (CFA injected but did not develop AA, age 18-22 months, n = 17 had a mean percent of total body weight loss of 11% compared to mean percent of body weight loss of 13% in the control group (saline injected, age 18-22 months, n = 16) but the difference was not statistically significant. * P< 0.0001 in young rats with AAversus control.

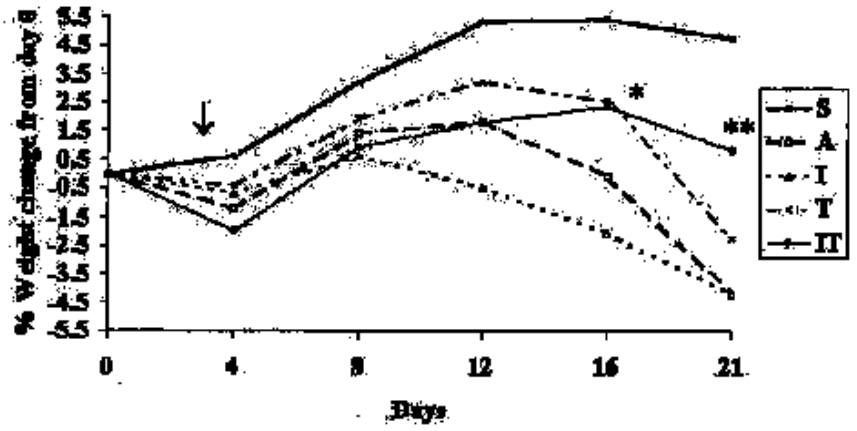

Fig. 3. TNF and IL-1 inhibitors prevent loss of body weight change in rats with adjuvant arthritis. Percentage of weight change in five groups of rats on days 4, 8, 12, 16 and 21 compared to day 0. Arrow points to day of starting treatments. Group S (n = 66) is the control group injected with saline on day 0. All other groups were injected with CFA on day 0. Group A (n = 78) received no treatment whereas Group I (n = 18) was treated with IL-1 Ra. Group T (n = 27) was treated with sTNFrI. Group IT(n = 8) was treated with both IL-1 Ra and sTNFrI. Error bars are omitted for the clarity of the figure.
* P< 0.01 (Group ITversus other groups). ** P< 0.002 (Group ITversus other groups).

but not given any treatment was 3.2 ± 0.3, whereas the mean joint scores in Group I (n =8 rats out of the original 18 rats in the group) was 8.6 ±1.4; in Group T (n=27) 6.8±0.8; and in Group IT (n=8) 6±1.4 ($p < 0.0001$ for groups I, T and ITvs. group A, $p \geq 0.05$ among groups I, T and IT).

Discussion

Adjuvant arthritis in Lewis rats has been used as a model for RA for many years, and a previous study demonstrated that AA is a model for inflammatory cachexia as well (1-2). In order to study the role of IL-1 and TNF in AA, previous studies have used cytokine inhibitors after rats have developed clinical AA (3-5). The purpose of our study was to determine whether blocking IL-1 and TNF after CFA injection would prevent susceptible animals from developing cachexia. We took advantage of the large sample size accrued in our control groups over a two-year period (young saline-injected animals and young CFA-injected animals) in order to pool our results across several experiments to maximize statistical power. These experiments were conducted in littermate animals using identical cage and diet conditions, and a single lot of adjuvant and mineral oil, by the same investigators, and analyzed by the same methods. Therefore, the present results benefit from more stable estimates of effect size as a result of the large sample size.

To determine the effects of age on inflammatory cachexia we compared joint swelling in young and old rats following CFA injection. In *Experiment 1*, we established that Lewis rats with an age range of 2-6 months are more susceptible to AA then rats with an age range of 18-22 months. As expected, the severity of AA in the young group correlated with the severity of weight loss when compared to a control group of saline injected rats. Although older rats showed a loss of body weight despite a lack of clinical arthritis, old rats in the control group had similar loss of weight. These experiments suggest that the mechanisms underlying the loss of muscle mass with age are overlapping those presiding at the loss of muscle mass induced by inflammation at young age. The present results therefore support the hypothesis that the loss of muscle mass associated with rheumatoid arthritis is not merely the extension of an intense local inflammatory process in the nearby joint. Because TNF and IL-1 are important mediators of joint inflammation, we aimed at elucidating whether TNF and IL-1 are required for the loss of body weight seen in a model of adjuvant arthritis. Since young Lewis rats aged 2-6 months had developed more severe AAwith inflammatory cachexia, we selected young Lewis rats with an age range of 3-5 months to study the potential preventive benefits of blocking IL-1 and/or TNF in terms of inflammatory cachexia.

The results of *Experiment 2* showed dissociation between the effects of blocking TNF and/or IL-1 on the severity of arthritis and the effects on weight loss. Blocking both cytokines showed a

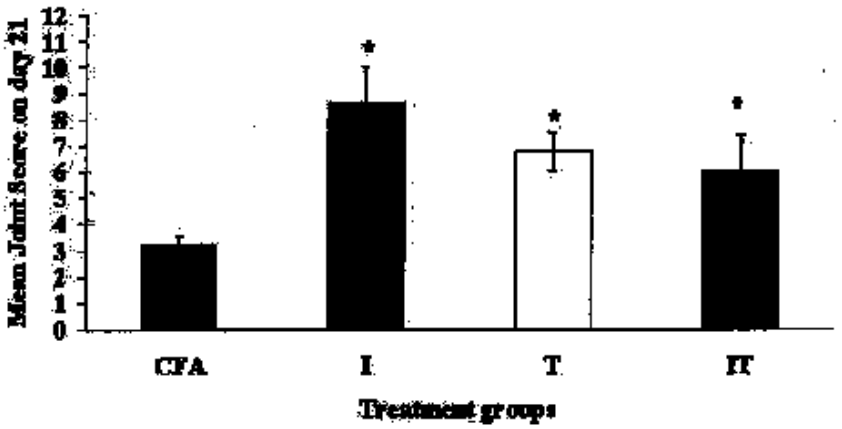

Fig. 4. Severity of AAin relation to cytokine blockade prior to developing clinical arthritis. * P < 0.0001 for groups I (treated with IL-1 Ra), T (treated with sTNFrI) and IT(treated with both IL-1 Ra and sTNFrI) versus untreated group of CFAinjected rats.

potential benefits on total body weight loss. However, this was paradoxically accompanied by worsened joint scores. We cannot fully explain the results we reached, but perhaps this phenomenon was a result of insufficient dosage of the medications we used in our experiment. For instance, McComb *et al*. (6) found that AA was attenuated by administration of sTNFrI that resulted in plasma levels of sTNFrI at 0.3–0.5 μg/ml. Further, a dose-dependent effect was observed since higher plasma levels resulted in a better inhibition of AA. In that study, delivery of the inhibitor started at the time of CFAinjection (6). Feige *et al.* (4) used various doses of IL-1Ra and PEGsTNFrI alone or together to study the effect of inhibiting IL-1 and/or TNF on severity of AA, loss of bone mineral density (BMD) and loss of body weight. Doses of IL-1Ra ranged between 0.2–5 mg/kg/hr via subcutaneous infusion for 7 days. Doses of PEGsTNFrI ranged between 0.25–4 mg/kg/day via subcutaneous infusion for 7 days. Cytokine inhibitors were administered after the clinical onset of AA, i.e., nine days after CFA injection. Changes in body weight of rats with AA during the 7 days of receiving one or both treatments were compared to the first day of receiving treatments (that was on day 10 after CFA injection). Results of the study showed partial alleviation of joint inflammation, loss of BMD and loss of body weight when each treatment was administered alone. Furthermore, combination of both treatments showed a synergistic capacity to inhibit these changes. Our data are in agreement with these results for body weight (i.e. for inflammatory cachexia) but not for severity of arthritis.

Bendele *et al*. (5) assessed the additive and/or synergistic effects of both treatments alone or together on established type II collagen-induced arthritis (CIA group) and developing adjuvant-induced arthritis (AA group). In the CIA group, IL-1Ra was administered starting on day 1 of arthritis using sustained-release delivery system of hyaluronic acid (HA) at a dose of 100 or 20 mg/kg sc for 6 days. PEG sTNFrI was administered intraperitoneally (ip) on days 1, 3 and 5 of clinical arthritis at a dose of 3, 1 or 0.3 mg/kg. In the AA group, the IL-1Ra in HA was given starting day 8 post-adjuvant injection using a dose of 100 mg/kg sc and continued through day 13. The PEGsTNFrI was administered using a dose of 3 or 1mg/kg ip on days 9, 11 and 13. In both groups the treatments were given alone or in combination with each other. Combination therapy (IL-1Ra 100 mg/kg + PEGsTNFrI 3 mg/kg) in the CIA group produced significant additive benefits for both body weight gain and ankle swelling, with the final measurements of ankle swelling being similar to normal rats. IL-1Ra at a dose of 100 mg/kg alone provided beneficial effects on ankle swelling while 20 mg/kg had minimal effect. Neither dose of IL-1Ra showed any beneficial effect on body weight gain. Similarly, there were no beneficial effects of any dose of PEG sTNFrI on body weight gain, despite dose-related inhibition of paw swelling, final paw weight and total histological scores for ankle joints.

In a study by Williams *et al*. (7), the effects of antibodies against TNF-α and IL-1 were evaluated using a model of collagen-induced arthritis. The results demonstrated that blockade of TNF-α or IL-1 before disease onset delayed but did not prevent the induction of arthritis. When anti-TNF-α, anti-IL-1β, or anti-IL-1R (which blocks both IL-1 α and β activities) were used after onset of arthritis they showed positive results in reducing the severity of arthritis, but changes in weight were not reported.

The beneficial effects reported in previous studies on joint score and severity of arthritis were not observed in any group of animals in our experiment, including the group that showed benefit in terms of weight loss. Paradoxically, these groups had worse mean joint scores compared to the group of young rats with AA but not treated with any cytokine blockade. To better understand the role of TNF-α and IL-1 in the different stages of inflammatory cachexia and AA, it may be important to study the kinetics of cytokine production by circulating and infiltrated leukocytes, and kinetics of cytokine gene expression in skeletal muscle, where the inflammatory cachexia is actually taking place. Nevertheless, the current experiments provide further evidence of the complexity of this process. In addition, we have new evidence of the effects of age on AA: young rats are more susceptible then old rats to AA and associated inflammatory cachexia.

References

1. ROUBENOFF R, ROUBENOFF RA, KEHAYIAS JJ *et al*.: Rheumatoid cachexia: Cytokine-driven hypermetabolism accompanying reduced body cell mass in chronic inflammation. *J Clin Invest* 1994; 93: 2379-86.
2. ROUBENOFF R, FREEMAN LM, SMITH DE, ABAD LW, DINARELLO CA, KEHAYIAS JJ: Adjuvant arthritis as a model of inflammatory cachexia. *Arthritis Rheum* 1997; 40: 534-9.

3. EISENBERG SP, EVANS RJ, AREND WP: Primary structure and functional expression from complementary DNAof a human interleukin-1 receptor antagonist. *Nature* 1990; 343: 341-5.
4. FEIGE U, HU YL, GASSER J, CAMPAGNUOLO G, MUNYAKAZI L, BOLON B: Anti-interleukin-1 and anti-tumor necrosis factor-α synergistically inhibit adjuvant arthritis in Lewis rats. *Cell Mol Life Sci* 2000; 57:1457-1470.
5. BENDELE AM, CHLIPALA ES, SCHERRER J *et al*.: 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. *Arthritis Rheum* 2000; 43: 2648-59.
6. MCCOMB J, GOULD T, CHLIPALA E *et al*.: Anti-arthritic activity of soluble tumor necrosis factor receptor type I form in adjuvant arthritis: correlation of plasma levels with efficacy. *J Rheumatol* 1999; 26: 1347-51.
7. WILLIAMS RO, MARINOVA-MUTAFCHIEVA L, FELDMANN M, MAINI RN: Evaluation of TNF-alpha and IL-1 blockade in collagen-induced arthritis and comparison with combined anti-TNF-alpha/anti-CD4 therapy. *J Immunol* 2000; 165: 7240-5.