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Modulation of TNF-α, TNF-α receptors and IL-6 after treatment with AM3 in professional cyclists

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***Aim*. Changes in IL-6, TNF-α, and TNF-α receptors - sTNFRI and sTNFRII - were evaluated in a group of professional cyclists treated with immunomodulator AM3 (Inmunoferón®) for 6 months of training and competition.**

***Methods*. Sixteen male professional cyclists with a similar training program participated in the study which was designed as a randomized, placebo-controlled, double-blind clinical trial. Venous blood samples were collected in basal conditions, before beginning the supplementation program, and after 90 and 180 days of training and competition season.**

***Results*. No significant differences in biochemical parameters or in IL-6 were evidenced between placebo and AM3-treated groups throughout the study. Plasma TNF-α levels significantly decreased (P<0.05) after 90 days of training in the AM3 treated group. TNF-α receptors increased during training season in both placebo and AM3 treated groups, although the increase was significantly higher (P<0.05) in the AM3 group with respect to the placebo group.**

***Conclusion*. The changes produced by regular training and competition were modified throughout the season by AM3 treatment which could reduce the inflammatory response to excessive exercise.**

KEY WORDS: Cytokines - Exercise - Inflammation.

There is a consensus about the anti-inflammatory effects of regular acute exercise.[1, 2] IL6 is the first cytokine that increases after exercise independently of the existence of muscle damage.[3] IL6 induces an anti-inflammatory environment by promoting the synthesis of the anti-inflammatory cytokines such as IL10 and cytokine inhibitors such as IL-1ra.[3] This response to exercise markedly differs from the cytokine release induced by infection, characterized by elevation in plasma levels of the classical proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and IL1.[3] However, it has also been reported little increases in TNF-α and IL1 after highly strenuous, prolonged exercise such as a marathon race.[4, 5] It was hypothesized that the ultra-structural damage of muscle tissue is a potential stimuli for the production and release of proinflammatory cytokines.[4-7] The cytokine response was suggested to play a role in muscle reconstruction after strenuous exercise and in the development of tolerance to ROS-induced muscle damage.[8, 9] The functional complexity of the cytokine network is expanded by the involvement of soluble receptors of cytokines.[10, 11] A clear example of this balanced signalization system is TNF-α and its two soluble receptors, sTNFRI and sTNFRII, which have the ability to block TNF-α biological activity.[10]

A continuous damaging stimuli induced by excessive training and competition bring about inflamma-

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tion, tissue damage and fatigue, which diminish recovery and performance in athletes.[12, 13] In this sense, professional cyclists perform large volume of exercise in training and during races, covering around 25,000 km in a season. Several authors reported high concentrations of pro-inflammatory cytokines to be correlated with over-training and reduced physical performance.[14, 15] Furthermore, disturbances in the immune system seem to be related in a higher risk for upper respiratory tract infections.[16, 17] The balanced modulation of cytokine-producing and cytokine-responding active immune cells is a promising medical field.[18] AM3 (Inmunoferón®) is an oral polysaccharide/protein immunomodulator purified from *Candida utilis* with regulatory effects on the production of several cytokines.[19-21] AM3 exerts its regulatory effect on TNF-α expression through a hypothalamo-pituitary-adrenal-dependent mechanism.[22] AM3 stimulates monocyte derived dendritic cells to increase the proliferation of allogenic T suggesting that AM3 might be useful in regulating immune responses in pathophysiological situations requiring dendritic cell maturation.[21] In this sense, the changes related to high performance sport were reported in terms of the effects of AM3 on the immune system, with a protective action against cellular stress.[23] However, these studies were carried out during a short period of time.

The aim of the present study was to determine the effects of the competitive cycling season and the immunomodulatory effects of AM3, developing a randomized, placebo-controlled, double-blind clinical trial, on serum levels of proinflammatory cytokines and anti-inflammatory receptors.

Materials and methods

Sixteen male professional cyclists (age 21-28 years) volunteered to participate in the study. Participants included in the study had performed more than three years of professional cycling competition and followed a similar training program. Participants were informed of the purpose of the study and the possible risks involved before giving their written consent to participate. Inclusion criteria for participation in the study included non-smoking status, absence of previously identified allergies to AM3 (Inmunoferón®) (taken in pill form, every day 30 min before breakfast), no infection one month prior to the study and the absence of chronic diseases. Participants that received immunosuppressants, immunomodulators, cimetidine, or any other medication considered to modify the immune response, were excluded from the study. A prior physical examination including an electrocardiographic evaluation and a blood test (hematological and serum biochemical parameters), performed following the International Cycling Union (ICU) protocols, ensured that each participant was in good health. The study protocol complied with the Declaration of Helsinki and was approved by the Research and Ethics Committee of Soria University (Spain).

Cyclists, components of the same professional cycling team, were randomly and double-blind treated with either AM3 (3 g/day) or an indistinguishable placebo during 180 days. The compliance of the participants was monitored by one of the members of the sport medical service of the cyclist team. Since the medical service worked daily with the subjects, no subject's data had to be removed due to non-compliance. Anthropometric and physiological characteristics of participants are shown in Table I and no significant differences were reported between placebo and AM3-treatred groups. Peripheral venous blood samples were collected in basal conditions, before the beginning the supplementation program, and after 90 and 180 days of training and competition. 90 and 180 day samples were taken 24 hours after the last training season. The clinical examination was also repeated during the experimental procedure, after 90 and 180 days, to monitor the healthy status.

The study was initiated at the beginning of the competitive racing season. Participants were training regularly an average of 600 km per week. Three weeks before the beginning of the research, each cyclist reported to the laboratory and took part in an incremental maximal cycling test. A mechanically braked cycle ergometer (Monark 818 E, Varberg, Sweden) adapted with a racing saddle, drop handlebars and clip-in pedals were used. The test started with an initial resistance of 110 W, with further increments of 35 W every 3 min. Participants kept a constant 75 rpm pedal cadence with the help of a metronome. Testing concluded when the required pedal cadence was no longer maintained by the cyclist. Heart rate was recorded at 5-s intervals through-

out the test (Polar S720i, Polar Electro Oy, Finland). Gas-exchange data were continuously monitored with a breath-by-breath metabolic cart (CPX-Plus, Medical Graphics Corporation, St. Paul, Minnesota, USA). Maximal power (W-max) was determined as the highest workload a cyclist could maintain for a complete 3-min period.

Cyclists followed a controlled diet and were instructed to ingest a standardized food and fluid plan based on individual body mass during training and preceding each race or laboratory test. Food intake was not allowed during the 3 h prior to the race. The diet was constantly supervised by the team.

Serum levels of urea, glucose, creatinine, total proteins, cortisol and the activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were made using commercial clinical kits in an autoanalyser system (Technicon DAX System).

Serum levels of IL-6, TNF-α, sTNFRI and sTNFRII were determined using a commercially available enzyme immunoassay kit (R&D Systems, Inc. MN, USA). All experiments were conducted in duplicate. A standard curve was obtained based on the standards provided by the manufacturer. Results were expressed as concentration of cytokines (in pg/ml) read from the standard curve. Values obtained below the standard range were considered to be non-measurable. Detection limits of the IL-6, TNF-α, sTNFRI and sTNFRII test kits were 0.094, 0.12, 3.0 and 1.0 pg/ml, respectively.

Statistical analysis

All analyses were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Results are expressed as mean±standard deviation and $P<0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were AM3 supplementation and time. The sets of data in which there was a significant interaction between both analyzed factors were tested by the ANOVA one-way test. When significant effects of supplementation or time factor were found, a Student's *t* test for paired data was used to determine the differences between the groups involved.

TABLE I.—*Anthropometric and physiological characteristics of participants.*

Subject characteristics	Mean±SEM
Mass (kg)	68.1±3.9
Height (cm)	178±4
Body fat (%)	7.0±0.3
VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$)	79.3±1.5
HR_{max} (bpm)	189±3
VO_2 RCT ($mL \cdot kg^{-1} \cdot min^{-1}$)	65.4±1.9
HR RCT (bpm)	169±2
VO_2 VT ($mL \cdot kg^{-1} \cdot min^{-1}$)	53.4±1.1
HR VT (bpm)	139±1
W-max (wats)	402±53

VO_{2max}: maximal oxygen uptake; HR_{max}: maximal heart rate; VT: ventilatory threshold; RCT: respiratory compensation threshold; W-max: maximal power.

Results

Serum concentrations of urea, glucose, ALT, AST, ALP, creatinine, LDH, total proteins, and cortisol measured in the placebo and in the AM3 treated groups are presented in (Table II). No significant differences between groups were observed in any of the parameters studied at baseline. No significant differences were evidenced between placebo and AM3 groups in any of the biochemical parameters studied. The plasma levels of urea and ALP after 90 days were significantly higher than at baseline situation in both placebo and AM3 groups, but these levels returned to baseline values in 180 day samples. The plasma activities of LDH, ALT and AST and the creatinine levels tend to decrease along the study; they were significantly lower after 180 days of study than at baseline. The GGT plasma activity was lower at 90 days than at baseline, but returns to it value at 180 days. No significant changes were observed in cortisol and plasma protein levels after treatment with AM3 or placebo during the study.

Neither training nor AM3 treatment affects the serum IL-6 levels (Table III). IL-6 showed similar baseline values in AM3 supplemented and control groups and no significant differences were found between the placebo and AM3-treated groups during 6 months. However, the TNF-a levels presented significant differences between groups and along the training season. TNF-α levels at 90 days of training were significantly lower than baseline in the AM3 supplemented group whereas in the placebo group the differences were not statistically significant. TNF-α

TABLE II.—*Biochemical characteristics of cyclists according to International Cyclist Union suggestions.*

Parameter	Baseline		90 days of treatment		180 days of treatment	
	Placebo	AM3	Placebo	AM3	Placebo	AM3
Glucose (mg/dL)	74.5 (71-81)	76.5 (72-82)	73.5 (59.2-81.5)	75 (69-83)	81 # (78-89)	78.5 (71.7-85)
Urea (mg/dL)	35.5 (34-39.5)	36.5 (34-41.5)	56 * (43-60)	51.5* (43.7-66.5)	39 # (32-45)	35.5 # (28.7-44.7)
Creatinine (mg/dL)	0.94 (0.86-0.98)	0.92 (0.83-0.97)	0.98 (0.53-1.1)	1.03 (0.86-1.17)	0.7 *# (0.68-0.88)	0.79 (0.77-0.89)
AST (U/L)	31.5 (25-37)	28.5 (22-35)	31 (24.2-38.7)	28.5 (23.5-42.5)	24 # (20-28)	22.5 (18.7-32.0)
ALT (U/L)	31.5 (28-41)	30.5 (27-40)	27 (16.7-29.5)	26.5 (21.5-38.7)	20 * (10-28)	24 (13.2-31)
GGT (U/L)	19.5 (17.8-21)	19.7 (17-21)	12.5 * (8.7-15.7)	14.5 (11-27.2)	19 (18-32)	17 # (14.2-23.2)
LDH (U/L)	490 (459-516)	481 (426-510)	370 * (340-415)	340* (293-451)	304 * (268-335)	316 * (301-446)
ALP (U/L)	53 (45-63)	51.5 (43-62)	135 * (107-138)	125 * (107-172)	92 * (88-164)	100 *# (80.5-112)
Total protein (g/dL)	7.1 (7-7.65)	7.05 (7-7.21)	6.76 (6.48-7.08)	6.98 (6.53-7.5)	6.87 (6.39-7.35)	7.05 (6.9-7.25)
Cortisol (μg/dL)	21.4 (12.6-26.4)	21.7 (13.8-27.5)	26.6 (15.8-32.7)	19.5 (15.3-23.7)	24.6 (13.7-35.6)	20.1 (12.9-27.4)

Biochemical parameters determined before the beginning of the study and after 90 and 180 days of AM3 treatment. (*) Indicates significant differences respect to baseline. (#) Indicates significant differences between 90 and 180 days of treatment. No significant differences were determined between placebo and supplemented groups. Values (n=8 each group) are mean and minimum and maximum range.

TABLE III.—*Changes in IL-6, TNFα and its receptors (sTNFRI and sTNFRII).*

	Baseline (pg/mL)		90 days of treatment (pg/mL)		180 days of treatment (pg/mL)	
	Placebo	AM3	Placebo	AM3	Placebo	AM3
IL-6	1.52±0.72	1.42±0.65	2.13±1.07	2.08±0.85	2.11±0.98	1.98±0.71
TNF-α	4.61±1.76	4.57±1.98	2.78±1.48	2.35±1.15 *	6.01±4.79 *#	4.00±2.60 #&
sTNFRI	954±220	939±218	1010±114	1236±202 *&	1081±167 #	1115±298 *&
sTNFRII	1724±385	1657±394	1694±244	1954±73 *	1783±298 #	1787±304 *

IL-6, TNF-a and its receptors (sTNFRI and sTNFRII) were determined before the beginning of the study and after 90 and 180 days of AM3 treatment. No significant differences were evidenced between controls and cyclists at baseline. (*) Indicates significant differences respect to baseline. (#) Indicates significant differences between 90 and 180 days of treatment. (&) Indicates significant differences between placebo and supplemented groups. Values (n=8 each group) are expressed as mean±standard deviation.

levels returned to baseline levels after 180 days of AM3 treatment, whereas in the placebo group, TNF-α levels significantly increased reaching higher levels than the measured at baseline. TNF-α receptors (sTNFRI and sTNFRII) showed similar baseline values in all groups studied (Table III). sTNFRI and sTNFRII levels significantly increased (P<0.05) after 90 of training in the AM3 group compared with baseline values of these receptors, whereas in the placebo group baseline values were maintained. However, both receptors showed higher levels after 180 days with respect to 90 days in the placebo group. sTNFRI was significantly (P<0.05) higher in the AM3 group with respect to the placebo group in both 90 and 180 day tests. No significant differences were evidenced in the sTNFRII levels between placebo-and AM3-treated groups in any moment of training season.

Discussion

It is well recognized that the excessive training stress, associated with insufficient rest and recov-

ery, may induce acute local inflammatory responses in working skeletal muscle that may evolve into chronic inflammation and produce systemic inflammation.[3, 14] The major finding of this study was the rise in serum levels of TNF-α receptors I and II (TNFRI and II) after 90 and 180 days of treatment with the immunomodulator AM3. TNF-α receptors followed a similar behavior in both studied groups, although the increases were less pronounced in the placebo group. TNF-α serum levels increased after 180 days of training and the AM3 treatment avoided this response. The present results also shown that nor the cyclist training nor the AM3 treatment reported significant effects on serum IL-6 and probably as a result of muscular adaptation of cyclists to training. Moreover, general serum biochemical parameters showed a normal response throughout the study suggesting the absence of any pathology in cyclist during the intervention period. Moreover, it is well established that regular endurance exercise results in adaptations in the skeletal muscle antioxidant capacity, which protects myocytes against the deleterious effects of oxidants and prevents extensive cellular damage.[24, 25] The beneficial effects of training on the markers of cellular damage are well established.[26] In the present results, the training and competition season tended to decrease the markers of liver and muscle damage, especially after 180 days of training, although, the treatment with AM3 not alters these adaptive effects of training. AM3 has been shown to exert a wide range of regulatory effects on innate and adaptive immunity in experimental and clinical models of inflammation and disease. AM3 has been reported to reduce the levels of serum TNF-α after administration of LPS [19, 27] and to normalize the decreased activity of polymorphonuclear and NK cells and macrophages of patients with chronic obstructive pulmonary disease (COPD).[28, 29] The absence of great differences after treatment with AM3 could be related to seasonally-induced changes and adaptation in cytokine concentrations and the good health status of participants.

IL-6 is a pleiotropic cytokine, which exerts diverse activities such as modulation and regulation of immune system responses, playing a central role in the cytokine cascade after exercise.[8, 30] Furthermore, IL-6 has been proposed as a glucose regulator during prolonged exercise, all of which may impact to the trained athlete.[31] Regular training and competition can help the adaptation of the immune system, and can modulate its response. Transcription and release of IL-6 are primarily regulated by an altered intramuscular milieu in response to exercise and, it has been pointed out that basal IL6 levels are reduced in response to training.[24, 32] However, in the present study, plasma levels were maintained at the baseline values during the training season analyzed. No significant effects were evidenced by AM3 treatment in the basal levels of IL-6 after 90 and 180 days of training, but in a previous study, we reported that AM3 supplementation during 65 days can moderate the rise in serum levels of muscle damage markers and in IL-6 after acute and strenuous exercise.[7, 23] The observed response could be associated with the stress levels induced not only by the training but also by the competition in which participate the cyclists.

TNF-α is a pro-inflammatory cytokine whose levels have been reported to rise in plasma after exhaustive exercise,[33] whereas, no variations in the blood levels of this cytokine have been evidenced in moderate or less intense exercise.[34] The present results showed that basal circulating levels of TNF-α tended to decrease at 90 days of training but significantly increased after 180 days of training and the AM3 treatment influenced this pattern. The anti-inflammatory effects of exercise could be responsible of the lower pro inflammatory status of the sportsmen after 90 days of training, although the more inflammatory status in these sportsmen after 180 days of training/competition could be a response to the excessive and repetitive practice of exercise.[1] The treatment with AM3 maintained baseline TNF-α serum levels after 180 days of training in a values significantly lower than placebo at this time of training and competition. These results are in accordance with an experimental study performed in mice, where AM3 has a clear inhibitory effect on TNF-α production in peritoneal macrophages.[19] The TNF-α response evidenced in the present results were accompanied with a rise in serum levels of TNF-α soluble receptors. In fact, the anti-inflammatory effect of acute exercise is associated with the secretion of soluble TNF-α receptors, IL-10 and IL-1ra.[35, 36] IL-1ra, IL-10 and soluble TNF-α receptors exert their respective anti-inflammatory effects by inhibiting signal transduction via the IL-1 receptor,[37] inhibiting cytokine gene expression and production in mononuclear cells [38] and binding and neutralizing circulating and membrane-

bound TNF-α.[39] The effects of AM3 evidenced in the present study could support an anti-inflammatrory and protective effects of AM3 against the action of TNF-α during long-period training.

Conclusions

The present study shows that the changes produced by regular training and competition can be modulated by AM3 treatment reducing the instauration of an inflammatory state. We suggest that an adequate training program accompanied by an effective therapeutic use of an immunomodulator such as AM3 could keep athletes in a good state of inflammation. These results can help to advance our knowledge about strategies to reduce illness, mainly inflammation and muscular damage in athletes, overall when they are involved in a long season of training and competition.

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