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Development and Disappearance of Tolerance to Induction of Interferon and Tumor Necrosis Factor Response in Athletes Treated with Natural Immunostimulant

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Abstract. The effect of nonspecific immunostimulation was examined in 15 basketball players subjected to extensive physical effort. The Tołpa* Torf Preparation (TTP*), a natural immunostimulating drug, was applied orally, one 5 mg tablet daily, in two 21-day cycles, separated by 2-week hiatus. Blood samples were collected 4 times, after each of two TTP* cycles and after the first and second hiatus. Whole blood assay was used to determine the spontaneous and induced production of interferon (IFN) and tumor necrosis factor (TNF). The levels of the cytokines were measured by microbioassays. TTP* stimulated synthesis of IFN and TNF in the whole blood cultures. However, after the oral administraton of TTP* for 3 weeks the leukocytes of the athletes developed hyporeactivity to IFN induction by TTP* and to a lesser extent to another "superinducer" – a mixture of phytohemagglutinin and bacterial lipopolysaccharide. The hyporeactivity state disappeared spontaneously within 2 weeks. In contrast, the tolerance to TNF induction did not develop during the TTP* administration. The increase of immunoglobulins, mainly of IgM and IgG classes and an acute phase protein – α_1-antitrypsin, was observed at the late phase of the treatment. We suggest that the cytokine levels may be early markers for immunoprophylaxis. Furthermore, high production of IFN and TNF may be associated with extensive physical effort.

Key words: nonspecific immunostimulation; basketball players; interferon; tumor necrosis factor; whole blood assay.

Introduction

The sport medicine has several major goals. The dominant one is how an individual can reach a maximal fitness in an appropriate time period delineated by local or international plans of the competition games. More specifically, that means how to obtain good results in sports by applying legal and safe forms of stimulation and training and how to avoid infections and other untoward reactions, frequently connected with a transient immunosuppression developing after extensive physical effort[16].

It appears that the noninvasive immunoprophylaxis with well-tolerated nontoxic drugs, administered in short cycles in low doses and by oral route, may be the treatment of choice for sportsmen.

The present study was designed to test whether the Tołpa* Torf Preparation (TTP*) may be useful as an

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immunostimulating drug in the basketball players subjected to extensive training and frequent games. TTP* is a preparation of natural origin, registered in Poland for human use, mainly to prevent and treat various immune disorders[4, 5, 17]. Its toxicity is remarkably low and it is administered by oral route in tablets. Recently, we discovered that TTP* acts in human peripheral blood leukocytes as inducer of interferon (IFN), tumor necrosis factor (TNF) and other cytokines[12, 13]. To assess the immunotropic action of TTP* *in vivo* we have applied an assay measuring hyporeactivity to interferon induction which develops after long-term oral administration of TTP* in humans[13]. Furthermore, we have measured the TNF response of the athletes, the production of acute phase protein and immunoglobulins. We also describe the practical value of the markers for evaluation of the nonspecific immunostimulation[10].

Materials and Methods

Volunteers. Participants in this study were 15 male basketball players, aged 21 ± 2 years and of height 196 ± 3 cm. They were engaged in the First National Basketball League games and in the play-off. Medical examination showed, that they were in good health, non-smokers and taking no medicines except of necessary food additives, like vitamins, mineral salts etc. They were informed about the study and gave their consent.

Treatment. Tołpa* Torf Preparation (TTP* trade mark of Torf Corp., Wrocław) in tablets á 5 mg, was obtained from the producer. TTP* is the peat extract obtained through a patented procedure, followed by dehydration. It has been registered in Poland as an immunostimulant for use in humans. TTP* contains low molecular weight organic substances (aminoacids, aminosugars, huminic acid, uronic acids, short peptides) and mineral salts[4, 5, 17].

All athletes were taking TTP* orally, one 5 mg tablet daily, shortly after the period of training or game. One cycle of the treatment lasted 21 days and it was always followed by 14 days hiatus. We have named such application of TTP* the "3+2" protocol.

Blood collection. Blood samples were obtained by venipuncture in 10 ml disposable syringes with 10 U/ml heparin (Heparinum, Polfa, Warsaw, without preservatives). They were processed within approximately 2 h storage at room temperature.

Whole blood cultures (*WBC*). Heparinized blood samples were diluted 1: 10 in RPMI 1640 tissue culture medium supplemented with 2 mM L-glutamine and antibiotics (penicillin 10 U/ml and streptomycin 100 μg/ml). Next, they were distributed in 1 ml aliquots into 24-well flat bottom tissue culture plates (Costar) to which the cytokine inducers at the indicated concentrations, in volumes of 10 μl/ml were added. The control cultures were prepared without the inducers. Plates were incubated in a cell culture incubator at 37°C with 5% CO_2 atmosphere for 20 h. Thereafter, the cultures were cleared by centrifugation and supernatants were stored at +4°C. For the determination of TNF activity the maximum storage period was a few hours (to avoid loss of activity due to proteolysis) whereas the IFN antiviral activity could be assayed within one week without substantial loss of activity.

IFN assay. The confluent monolayers of A549 cells (human lung adenocarcinoma, ATCC, CCL 1185) were prepared in Costar microplates in Dulbecco modified minimal essential medium (DMEM), supplemented with 10% calf serum, L-glutamine and antibiotics. IFN--containing samples were diluted in separate plates and added to the cell monolayers. After incubation at 37°C for 20 h in the CO_2 incubator the cels were once washed with the medium and challenged with encephalomyocarditis virus (EMCV). The MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) method to measure the A549 cell kill in the ELISA scanner was used[2]. The end-point of IFN titration in the MTT assay (one unit) was taken as 25% reduction of the cell kill in comparison with control cells without virus or cell infected with 100 $TCID_{50}$ of EMCV[11-13]. The standards of IFN-α and IFN-γ were included in all assays. The solid phase immunoassay for detection of IFN-γ with two monoclonal antibodies anti-human IFN-γ and anti-immunoglobulin reagent was made as described by GAWLIKOWSKI[8].

TNF assay. The cytotoxic activity of TNF was measured in L_{929} cells according to FLICK and GIFFORD[6] with several modifications. The samples titrated in culture medium with the addition of actinomycin D (Sigma, 5 μg/ml) were added to monolayer cultures of L cells. The cultures were incubated at 37°C in 5% CO_2 in air for 20 h and cytotoxic effects were determined by microscopic examination and by the MTT method[12, 13]. One unit of TNF in our assay was equal to 100–200 pg/ml of TNF-α (Genentech Inc. S. San Francisco, USA).

Inducers and other reagents. Phytohemagglutinin (PHA-L, cat. No L-4144) and lipopolysaccharide (LPS from *E. coli* 055-B5, cat. No L-6018) were Sigma Cell Culture Reagents, St. Louis, MD, USA.

Three different batches of TTP* in powder prepared for laboratory studies by Dr. K. Witkiewicz at Torf Corp., Wrocław contained approximately 60% of mineral salts, mainly NaCl, 35% the organic substances and

5% of water. Minimal cytotoxic dose of TTP* for human leukocytes was from 2–3 mg/ml[12].

Blood examination was done using automatic hematological analyzer Coulter MAX-M. The blood proteins were determined by nephelometric kinetics methods using RA-1000 Technicon* apparatus[1, 3, 14] and commercial kits.

Statistical analysis. Parametric and non-parametric tests were used to compare the results. Student's *t*-test for independent samples and Wilcoxon test were used. Analyses were performed on CSS, Statistica, STAT Soft* Tulsa, OK, USA.

Results

Production of cytokines and development of tolerance

The group of 15 basketball players received two (3-week) cycles of TTP* separated by 2-week hiatus in April to May 1993 (periods A, B, C, D). For the technical reasons, connected with the program of training and games, the blood samples were drawn 2 days after the first cycle of TTP* (A) and next one day after the hiatus (B), and similarly after second TTP* cycle (C) and after second hiatus (D). Eight out of 15 volunteers donated blood 4 times, others gave blood 2–3 times.

All blood samples were subjected to the whole blood culture to assay the IFN and TNF responses to inducers. The cytokine levels were also determined in all plasma samples.

Figures 1 and 2 summarize the results of four WBC assays which showed the IFN and TNF responses after stimulation with 3 different batches of TTP* and with the "superinducer" – PHA+LPS.

The blood cultures incubated without the inducers served as controls of the levels of the "spontaneous" cytokine production.

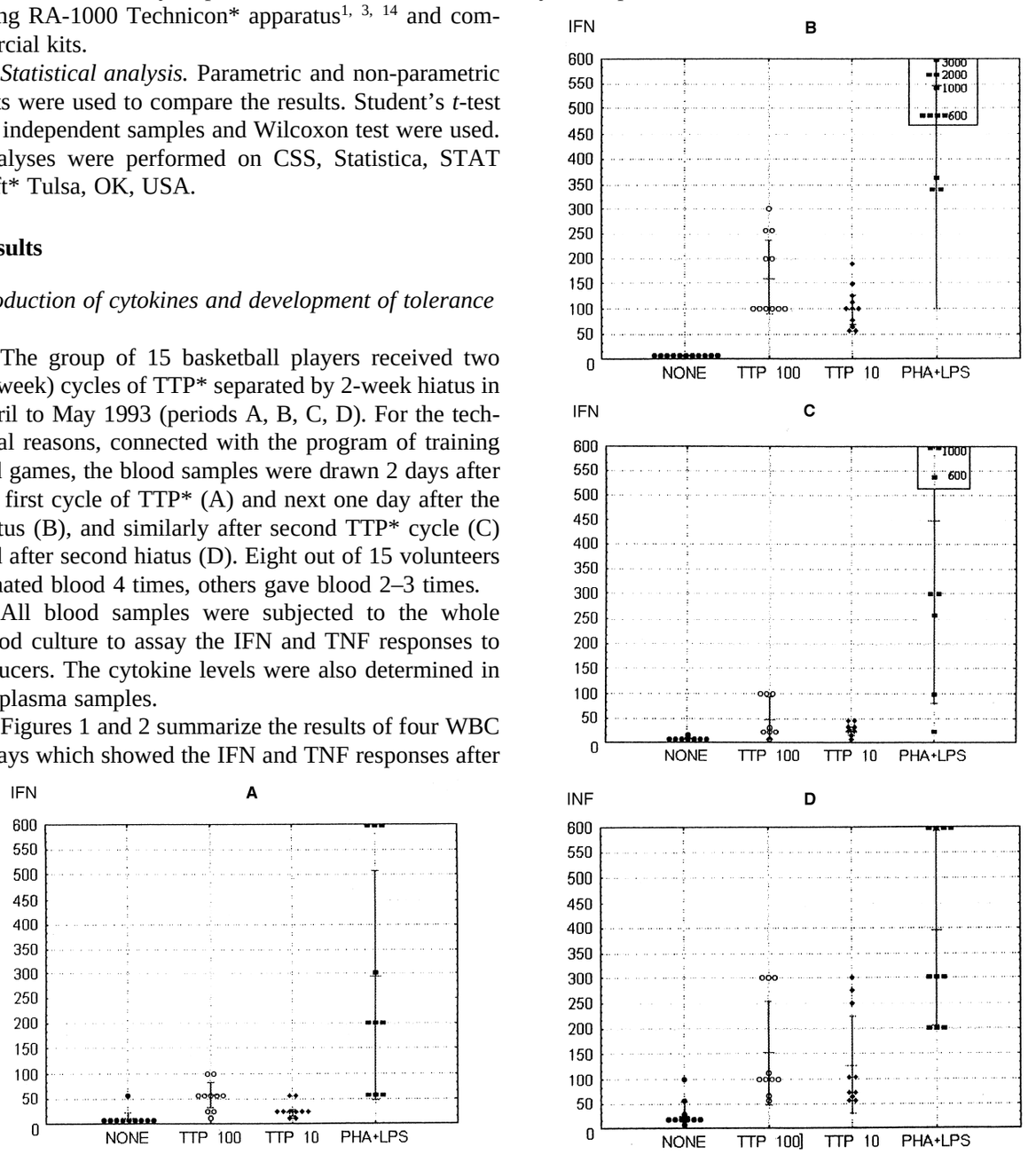

Fig. 1. Production of interferon (IFN) by whole blood leukocytes of basketball players after treatment with TTP* and after hiatus. A – the time period after the first 3-week cycle of TTP* administration, B – the time period after the first 2-week hiatus in the treatment, C – the time period after the second 3-week cycle of TTP* administration, D – The time period after the second 2-week hiatus in the treatment. A vs B statistically significant at $p<0.001$ for "TTP 100", $p<0.0001$ for "TTP 10" and $p<0.05$ for "PHA+LPS". B vs C statistically significant at $p<0.002$ for "TTP 100" and $p<0.001$ for "TTP 10". C vs D statistically significant at $p<0.02$ for "TTP 100" and $p<0.01$ for "TTP 10". The comparisons not mentioned above were statistically not significant. Abscissa: cytokine inducers; ordinate: cytokine U/ml

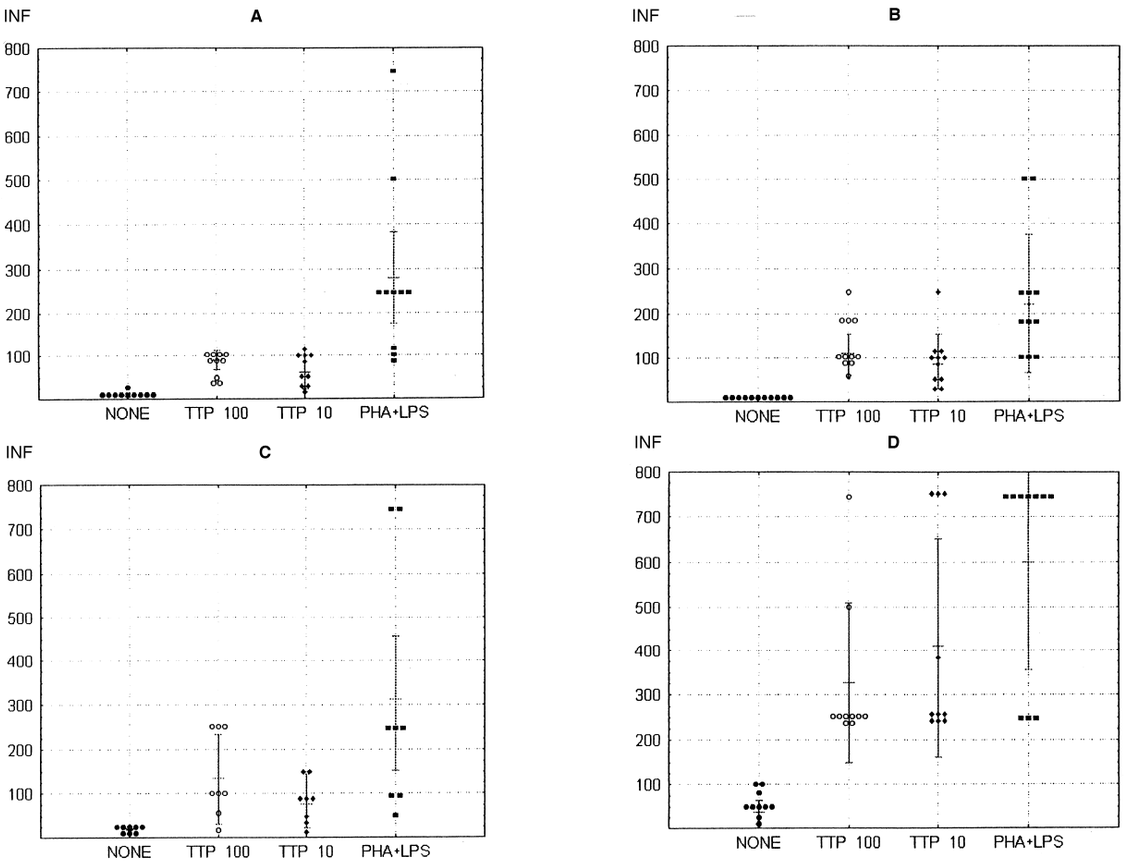

Fig. 2. Production of tumor necrosis factor (TNF) by whole blood leukocytes of basketball players after treatment with TTP* and after hiatus. For explanation see Fig. 1. A vs B statistically significant at $p<0.05$ for "none". B vs C statistically significant at $p<0.001$ for "none". C vs D statistically significant at $p<0.005$ for "none", $p<0.02$ for "TTP 100", $p<0.002$ for "TTP 10" and $p<0.05$ for "PHA+LPS". The comparison not mentioned above were statistically not significant

Table 1. IFN and TNF responses in the whole blood assay of healthy nontrained volunteers (controls)

Number of blood samples	Inducers (μg/ml)	Cytokines (U/ml)			
		IFN		TNF	
		mean titer ± SD	range	mean titer ± SD	range
32	none	2.4 ± 3.2	<3–12	17.8 ± 43.5	<4–250
32	PHA+LPS (2+2)	202.9[a] ± 158.1	20–600	326.5[a] ± 390.1	26–1600
32	TTP* (100)	58.2[a] ± 80.5	<3–300	196.1[a] ± 251.4[a]	18–750

Blood samples of 20 healthy donors, who were not athletes, were assayed in the whole blood cultures for 20 h at 37°C.
IFN and TNF were measured by the bioassays.
[a] Significantly different from "none" at $p<0.0002$.

Table 2. Levels of IFN and TNF in plasma of the basketball players after TTP* treatment and after hiatus

Cycles	Cytokines (U/ml) in plasma samples	
	IFN[a]	TNF[a]
A – after first 3 weeks administration of TTP*	<3, <3, <3, <3, <3, <3, <3, <3, <3, <3	9, 9, 9, 9, 9, 9, 9, 9, 9, 18
B – after first 2 weeks hiatus	<3, <3, <3, <3, <3, <3, 6, <3, <3, <3, 6	6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6
C – after second 3 weeks administration of TTP*	<3, <3, <3, <3, 6, 3, 24, 3	18, 9, 18, 18, 9, 9, 9, 18
D – after second 2 weeks hiatus	24, 6, 18, 6, 18, <3, 24, 72, 18, <3	9, 9, 27, 9, 27, 27, 27, 27, 9, 9

C and D cycles coincided with the beginning of the competition games.
[a] Levels of IFN or TNF in the individual plasma donors. The sequence refers to paralel study of the same donor.

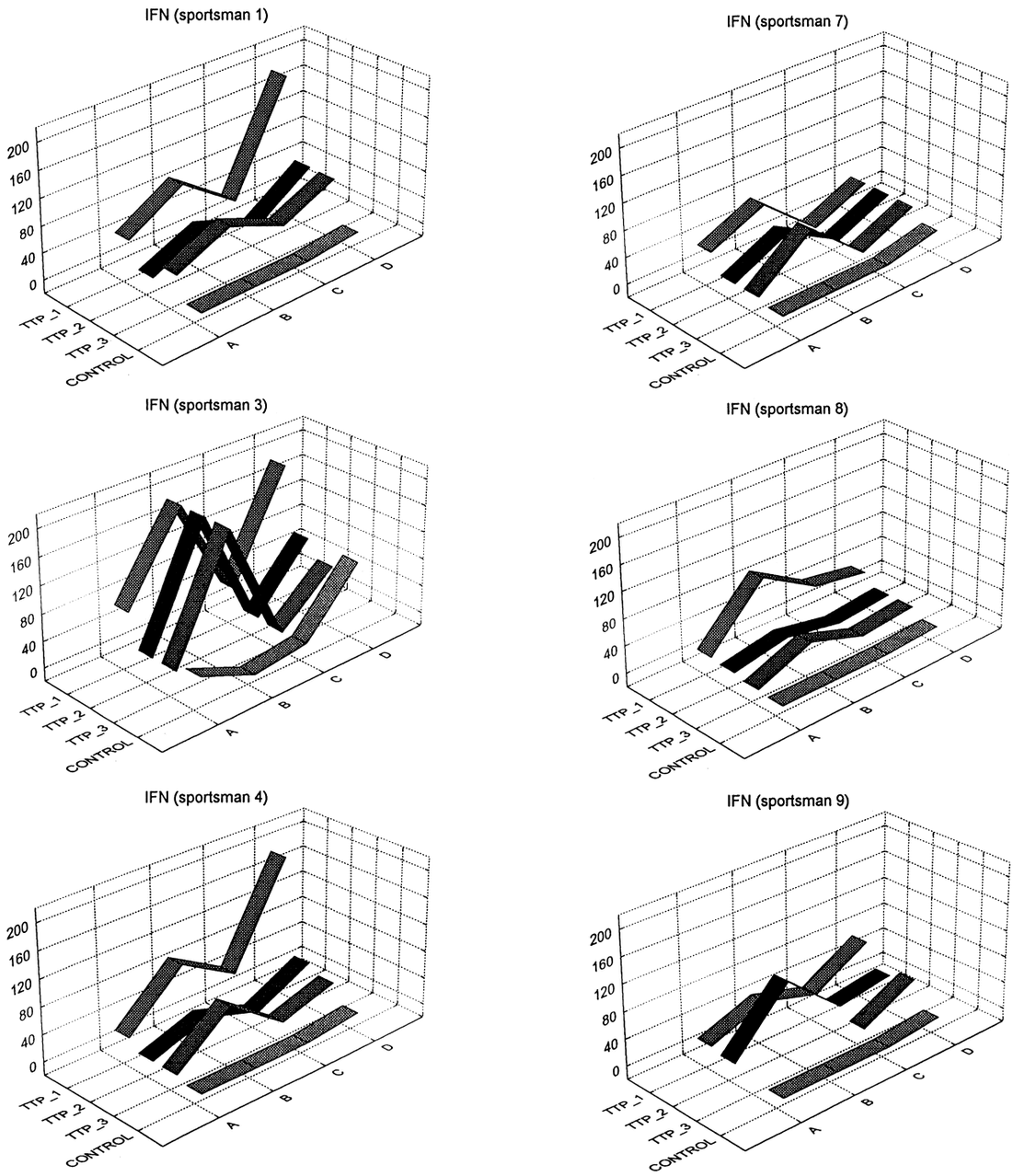

Fig. 3. Fluctuating curves of IFN production in 6 individual basketball players subjected to two 3-week cycles of immunostimulation with TTP* separated by two 2-week hiatus. A, B, C, D – time periods, see Fig. 1. For explanations see text

There was a marked IFN response to all inducers at the end of the first and second hiatus in the administration of TTP* (Fig. 1B and D). On the other hand, at the end of the first and second cycle of TTP* treatment the IFN response of whole blood leukocytes to TTP* and PHA+LPS was significantly reduced or even abolished (Fig. 1A and C). In contrast, Fig. 2 (A, B, C, D) shows that the TNF response to the inducers was almost the same either after TTP* administration or after the first hiatus, but except of the increased levels of TNF at D time period during finals (second hiatus).

Controls constituted 20 healthy nontrained individuals (4 females and 16 males) who donated 32 blood samples. They were investigated in the same way and with the same reagents as the basketball players. Results showed that their mean IFN and TNF responses were approximately the same as the responses of the athletes assayed during the hiatus in the TTP* admin-

Table 3. Hyporeactivity to IFN-γ induction measured in the whole blood culture supernatants by the microbioassay or by ELISA

Treatment	IFN inducer antiviral (U/ml)	IFN-γ – ELISA (pg/ml)
sportsman number 1		
TTP*, 3 weeks	none	<3	3.6
TTP*, 3 weeks	TTP*	96	942.0
TTP*, 3 weeks	PHA	600	3696.0
hiatus 2 weeks	none	<3	<1
hiatus 2 weeks	TTP*	96	2548.0
hiatus 2 weeks	PHA	2000	4482.0
sportsman number 8		
TTP*, 3 weeks	none	<3	0
TTP*, 3 weeks	TTP*	24	530.0
TTP*, 3 weeks	PHA	600	4529.0
hiatus 2 weeks	none	<3	143.0
hiatus 2 weeks	TTP*	180	2421.0
hiatus 2 weeks	PHA	3000	4348.0

Blood was collected at the end of the treatment with TTP* or after hiatus.
ELISA was performed as described by GAWLIKOWSKI[8].

istration. However, the variations of the cytokine responses were significantly lower among the athletes than among the nontrained controls (Table 1, Fig. 1 and 2).

The plasma levels of IFN and TNF were undetectable after the first and second cycles of TTP* and after the first hiatus. However, the elevated plasma levels of the cytokines were found after C and D cycles (Table 2). These time periods coincided with the finals of the first league competition games and with the highest physical effort and stress of the athletes.

We have also prepared graphs illustrating the individual IFN and TNF responses of 6 randomly selected basketball players after TTP* administration and after hiatus. The pattern of their responses to inducers were found to be almost identical – the low responses after TTP* administration and high after hiatus. However, the individuals differed from one to another in the amplitudes of responses. The best competitors were also the best IFN and TNF producers (Fig. 3).

We have previously found that TTP* may induce in peripheral blood leukocytes both IFN-α and IFN-γ distinguished by neutralization assays[12, 13]. In this study we used the specific ELISA assay for IFN-γ to measure the relative contribution of IFN-γ to the IFN antiviral activity after the TTP* administration and after hiatus in two selected basketball players. Table 3 shows that the levels of IFN-γ induced by TTP* after hiatus were significantly higher that after the TTP* administration. The differences between the levels of IFN-γ, induced by PHA+LPS after the TTP* treatment or after hiatus, were less impressive. Because 1 U of the antiviral activity of IFN-γ equals approximately 100 pg/ml of IFN-γ[8], it appears that the observed IFN antiviral activity is a result of action of several subtypes of IFN.

Other laboratory studies

To measure the biochemical effects of the extensive physical effort, the activity of acute phase protein – α_1-antitrypsin (AAT) was determined. Our previous studies showed that levels of AAT in serum, as well as urinary inhibitor (UTI) in urine, increase after the marathon run[1, 3, 14].

Here we found that levels of serum AAT significantly increased during the second time period (Fig. 4C and D) in comparison with lower levels during the first treatment and hiatus (Fig. 4A nad B). This was accompanied also by significant increase of IgM concentration (Fig. 4B, C and D).

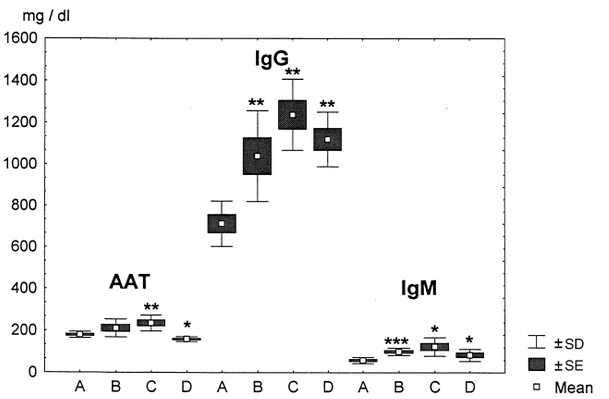

Fig. 4. Levels of α_1-antitrypsin activity (AAT) and immunoglobulins during the course of immunostimulation with TTP*. A, B, C, D – time periods, see Fig. 1. * Significant results at p≤0.05, ** significant results at p≤0.01, ***significant results at p≤0.005

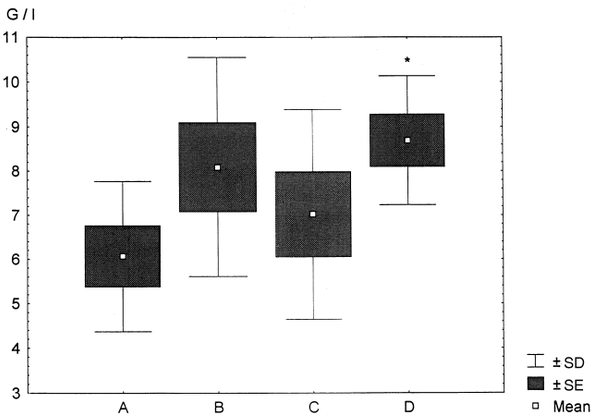

Fig. 5. Total number of white blood cells during the course of immunostimulation with TTP*. A, B, C, D – time periods, see Fig. 1. *Significant results

At the A, B, C, D time periods total peripheral blood leukocyte number was determined. The significant ($p<0.05$) increase in the leukocyte number was found at D time period (second hiatus), which was the time of finals of the competition games (Fig. 5).

Subjective observations of a masseur of the athletes

The masseur of the basketball team thoroughly collected information about the symptoms of illness, adverse reactions and other self evaluation of subjects during the whole time period of training, games and the treatment with TTP*.

At the first period of games (December 1992 to March 1993) two months before the administration of TTP*, the majority of the basketball players had either upper respiratory tract infections or other diseases which required a few days of retirement from the games. During the whole time of the TTP*treatment not a single case of respiratory disease was recorded. All athletes self evaluated their health as good and as many as 9 out of 15 expressed the opinion that their fitness is better than before the treatment. However, it has to be mentioned that the athletes were examined at the peak of their fitness when their ranking was made.

At the A, B, and C time periods of regular training, play-off and the games, the overall physical effort was approximately the same. However, D time period coincided with the break in TTP* administration and finals for the first league in basketball.

Discussion

The effects of nonspecific immunostimulation are difficult for objective assessment[10, 11]. However, we can state that the oral administration of the immunostimulating drug – TTP* in low doses, in two 3 week cycles, separated by 2-week hiatus (called shortly "3+2 schedule"), was very well tolerated by all basketball players. No untoward effects have been observed. During the experiment, which included the regular training program, a period of play-off and the final competition games no serious infections or even common colds were observed.

One can say that it could be a pure coincidence. However, we performed also the detailed laboratory study of blood components of the athletes and we measured the production of cytokines by the whole blood cultures. These were the objective parameters of the induced nonspecific immunostimulation.

We think that the most useful marker of immunostimulation by TTP* is the cyclic appearance and disappearance of hyporeactivity to IFN inducers in the peripheral blood leukocytes. After each of the two 3-week cycles of TTP* all subjects developed partial or even complete tolerance to IFN induction by 3 different batches of TTP* and in somewhat lesser extent – to the mixture of PHA and LPS (which was considered to be the "superinducer"). The tolerance was abrogated during 2-week hiatus[13, 15].

The tolerance to IFN induction was apparently due to administration of the immunostimulant, in our case to TTP* and not to high physical effort. During A, B and C time periods the rate of exercises was about the same, whereas at D period – the peak of games the IFN and TNF responses, were found to be maximal (Fig. 1 and 2).

It is characteristic that in the athletes the tolerance to TNF induction has not developed. This suggests that IFN and TNF have different mechanism of regulation of synthesis. TNF may even be regarded as a biologic substitute of IFN during the period of tolerance.

We have also found significant changes in some plasma proteins content during the late time period of the immunostimulation with TTP*. There was an increase in concentration of acute phase protein – AAT and immunoglobulins (IgG, IgM). One can conclude that in our study, as well as in other investigations, various immunostimulants at first stimulate the Th1 cell population, as well as various phagocytes, to produce different "inflammatory" and other cytokines. Thereafter, the reverse, homeostatic reactions are activated. The production of IFN (but not TNF) is switched-off whereas other inhibitory cytokines are switched-on. It is evidenced also by the increased production of acute phase protein and immunoglobulins[1, 3, 7, 9, 12, 14, 16].

At the period of competition games, connected with great stress, small levels of both IFN and TNF appeared in plasma of the basketball players spontaneously. In healthy subjects the circulation of the cytokines is a transient event, usually efficiently compensated by homeostatic mechanisms.

In summary, we think that the long-term immunostimulation with the non-toxic and non-hormonal oral drug TTP* is safe and well tolerated. We think that it is worthwhile to plan double-blind studies to find out whether the cyclic, oral, nonspecific immunostimulation may prevent infections and increase fitness of sportsmen subjected to high physical effort and stress.

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