ORIGINAL ARTICLE

Reversal in fatigued athletes of a defect in interferon γ secretion after administration of *Lactobacillus acidophilus*

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Br J Sports Med 2006;**40**:351–354. doi: 10.1136/bjsm.2005.024364

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Accepted 22 November 2005

Background: Fatigue and impaired performance in athletes is well recognised and has been loosely linked to ''overtraining''. Reduced concentration of IgA in the saliva and increased shedding of Epstein Barr virus (EBV) have been associated with intense training in elite athletes.
Objective: To determine whether athletes presenting with fatigue and impaired performance had an immune defect relevant to defective containment of EBV infection, and whether a probiotic preparation (*Lactobacillus acidophilus*) shown to enhance mucosal immunity in animal models could reverse any detected abnormality.
Results: The fatigued athletes had clinical characteristics consistent with re-activation of EBV infection and significantly ($p = 0.02$) less secretion of interferon (IFN) γ from blood CD4 positive T cells. After one month of daily capsules containing 2×10^{10} colony forming units of *L acidophilus*, secretion of IFNγ from T cells had increased significantly ($p = 0.01$) to levels found in healthy control athletes. A significant ($p = 0.03$) increase in salivary IFNγ concentrations in healthy control athletes after the one month course of *L acidophilus* demonstrated in man the capacity for this probiotic to enhance the mucosal IFNγ concentration.
Conclusion: This is the first evidence of a T cell defect in fatigued athletes, and of its reversal following probiotic therapy.

Impaired athletic performance and fatigue in well trained athletes and its relation to recurrent infection and reduced mucosal immunity has been the subject of scientific investigation.[1 2] Protracted training at an intense level can cause persistent suppression of salivary IgA concentrations.[3 4] Regression analysis revealed that the number of episodes of upper respiratory tract illness over a training session could be predicted in elite swimmers by both the preseason and the mean pretraining concentration of salivary IgA.[5] A recent study of the re-activation of Epstein Barr virus (EBV) infection in relation to upper respiratory tract illness in elite swimmers confirmed that the appearance of EBV DNA in saliva was associated with episodes of upper respiratory tract symptoms and was preceded by depressed concentrations of IgA in saliva.[6]

It is likely that intensive exercise (and other stressors) alter T cell control mechanisms which in turn suppress EBV specific cytotoxic T lymphocytes and secretory IgA, variables associated with control of EBV shedding in the upper respiratory tract.[6 7] Therapeutic attempts to limit EBV shedding and related symptoms have used antiviral agents.[7] An alternative mechanism to control EBV shedding could involve enhancement of mucosal T lymphocyte mediated immunity, to prevent expansion of the latent EBV infected B cell population. This cell population is considered to be the precursor to mucosal inflammation and clinical symptoms.[8] Recent murine studies have shown that selected isolates of *Lactobacillus acidophilus* can enhance T cell function and protect against mucosal infection,[9] indicating that probiotics may contribute to the management of subjects with defective, or exercise induced, mucosal T cell immunity.

The aims of this study were firstly to determine if immune variables differed between healthy and fatigued athletes, and secondly, to determine whether these immune variables changed after lactobacillus intervention therapy.

SUBJECTS AND METHODS

Subjects

This study was designed to evaluate the effects of *L acidophilus* LAFTI®L10 (DSM Food Specialties, Moorebank, NSW, Australia) on immunity in healthy and ''fatigued'' athletes. All athletes were well trained recreational athletes. The fatigued athletes were self referred to a medical sports clinic complaining of fatigue, recurrent sore throats, and impaired performance. The control group included healthy, well trained recreational athletes who volunteered for this study. Table 1 summarises the characteristics of both groups. The study was conducted as a pre-post intervention study.

EBV shedding in saliva was assessed using the real time polymerase chain reaction (PCR) technique[7] in 24 saliva samples collected from the eight seropositive fatigued subjects (three samples from each subject) taken before and after the course of *L acidophilus*. The treatment protocol consisted of four weeks of *L acidophilus*, taken as a daily capsule containing 2×10^{10} colony forming units. Six of 24 saliva samples (25%) from five of the eight (63%) seropositive subjects with fatigue had detectable EBV DNA before the probiotic treatment, whereas afterwards only one of 24 saliva samples (4%) had detectable EBV DNA. The viability of the *L acidophilus* preparation was monitored and validated throughout the period of the study.

Laboratory measures

Unstimulated, non-fasting, whole mixed saliva samples were collected into 5 ml plastic collection tubes and stored at −20℃ until analysis. Salivary IgA was measured by enzyme linked immunosorbent assay (ELISA),[10] and saliva osmolality by freezing point depression using an osmometer (The

Abbreviations: EBV, Epstein Barr virus; IFN, interferon; IL, interleukin; PCR, polymerase chain reaction

Table 1 Subject characteristics and association of upper respiratory illness (URI) with physical training in the healthy and fatigued athletes

Subjects' details	Healthy athletes	Fatigued athletes
Number studied	18	9
Male/female	11:7	6:3
Age (years)	26.1 (16.7 to 37.1)	24.7 (17 to 40.2)
Training level (hours/week)	10.7 (5 to 17)	20.9 (15 to 30)
VO_{2MAX} (ml/kg/min)	50.6 (35.1 to 68.3)	53.7 (44.9 to 68.4)
Fitness category*		
High (number in group)	12	6
Moderate (number in group)	6	3
Episodes of URI/year	1.7 (0 to 4)	3.5 (1 to 7)
Duration of URI episode (days)	5.2 (2 to 10)	13.4 (4 to 35)
Lost activity/episode (days)	1.9 (0 to 12)	15.8 (0 to 45)
Association of URI with intense training		
Never (number in group)	9	0
Sometimes (number in group)	7	3
Usually (number in group)	2	6
Sleep disturbance (number in group)	3	5
EBV serology (number positive in group)	12	8

Values are median (95% confidence interval).
*Fitness level determined from VO_{2MAX} test according to established formula.
EBV, Epstein Barr virus; VO_{2MAX}, maximum oxygen uptake.

Advanced Micro Osmometer, model 3300; Advanced Instruments Inc, Norwood, Massachusetts, USA). Whole blood cultures were performed to measure cytokine secretion (interleukin (IL) 4, interferon (IFN) γ, IL12) as previously described.[11] EBV DNA was measured by PCR.[7]

Statistical analysis

Statistical analyses were performed in Stata version 8. Data were checked for normality, and outliers and all continuous measures were found to have highly skewed distributions. Box-Cox power and log transformations (bcskew0 and lnskew0 respectively) were applied to obtain normally distributed variables. The transformations for IFNγ, IL12, and IL4 did not provide distributions appropriate for parametric analysis. IgA and IgA/osmolality ratios were transformed to an approximately normally distributed variable. However, IgA and IgA/osmolality ratios were analysed using non-parametric methods to be consistent with analyses for IFNγ, IL12, and IL4.

The pretreatment concentrations of salivary IgA and IFNγ and blood CD4 T cell secreted cytokines were compared between the healthy and fatigued groups of athletes using the Ranksum test for independent samples. Changes from pre to post treatment were compared separately for the healthy and fatigued groups, as well as for both groups combined using the Signrank test (for dependent or paired samples). Change scores were calculated by subtracting the pretreatment value from the post-treatment value for each subject for all outcomes of interest. To determine whether the probiotic effect differed between healthy and fatigued athletes, the differences were compared between healthy and fatigued groups using the Ranksum test. Significance was accepted at $p<0.05$.

RESULTS

Tables 2 and 3 show the concentrations of salivary IgA and IFNγ and whole blood cultured secreted cytokines IFNγ, IL4, and IL12 before and after *L acidophilus* treatment for the healthy and fatigued groups respectively. There were no significant differences between the healthy and fatigued athletes with respect to salivary IgA ($Z = -0.180$, $p = 0.86$), the IgA/osmolality ratio ($Z = -0.103$, $p = 0.92$), salivary IFNγ ($Z = -0.838$, $p = 0.40$), or for the secretion from whole blood cultures of IL12 ($Z = -1.132$, $p = 0.26$) and IL4 ($Z = 0.311$, $p = 0.76$). However, the secretion of IFNγ from blood CD4 T cells measured in whole blood culture was significantly lower in fatigued athletes than in the healthy controls ($Z = 2.291$, $p = 0.02$).

After four weeks of treatment with *L acidophilus*, the whole blood culture secretion of IFNγ was significantly higher after probiotic treatment ($Z = -2.384$, $p = 0.02$). Similarly, the increase in salivary IFNγ concentration approached significance ($Z = -0.878$, $p = 0.06$). There were no significant changes in the combined group for salivary IgA, salivary IgA/osmolality ratio, or secretion of IL4 or IL12 in whole blood cultures after probiotic therapy. There were no other

Table 2 Immune variables in 18 healthy athletes before and after one month of administration of *Lactobacillus acidophilus*

	Before		After		Signrank test	
	Median	95% CI	Median	95% CI	Z value	p Value
Saliva IgA (mg/l)	59	49 to 71	51	47 to 65	0.697	0.49
Saliva IgA/osmolality ratio	0.97	0.69 to 1.09	0.80	0.70 to 1.04	0.022	0.98
Saliva IFNγ (pg/ml)	10	8 to 21	15.1	8.8 to 23.0	−2.127	0.03
Whole blood culture secretion						
IFNγ (pg/ml)	11.9	7.8 to 57.8	26.8	12.2 to 43.3	−1.071	0.28
IL4 (pg/ml)	28.1	12.4 to 52.8	18	12.3 to 27.8	1.327	0.18
IL12 (pg/ml)	287.6	172.1 to 352.6	321.2	250.3 to 356.0	−0.240	0.81

IFN, Interferon; IL, interleukin.

Table 3 Immune variables in nine fatigued athletes before and after one month of administration of *Lactobacillus acidophilus*

	Before		After		Signrank test	
	Median	95% CI	Median	95% CI	Z value	p Value
Saliva IgA (mg/l)	56	47 to 103	70	32 to 81	0.140	0.89
Saliva IgA/osmolality ratio	0.78	0.75 to 1.33	0.86	0.47 to 0.97	0.178	0.86
Saliva IFNγ (pg/ml)	11.5	7.9 to 41.2	25.5	7.9 to 39.0	−0.415	0.68
Whole blood culture secretion						
IFNγ (pg/ml)	7.8	7.8 to 9.0	32.1	9.4 to 80.2	−2.611	0.01
IL4 (pg/ml)	20.4	9.8 to 97.7	35.4	15.8 to 101.6	−1.007	0.31
IL12 (pg/ml)	318.3	246.3 to 725.0	271.0	40.7 to 652.7	1.362	0.17

IFN, Interferon; IL, interleukin.

significant changes after the treatment. The changes did not differ between the healthy and fatigued athlete groups, for any variable, although the concentrations of salivary IFNγ after treatment were significantly higher in the healthy group (table 2) and for whole blood culture secretion of IFNγ in the fatigued group (table 3).

DISCUSSION

Athletes complaining of fatigue, particularly associated with recurrent infections and declining performance, had significantly less secretion of IFNγ from blood CD4 positive T cells in cultures than healthy control athletes. After treatment with *L acidophilus* there was a significant increase in secretion of whole blood IFNγ to levels similar to those found in the control athlete group. As IFNγ is a cytokine intricately linked to mechanisms of control of both virus shedding and disease re-activation, these observations are relevant to putative preventive intervention for relapsing EBV infection.

Review of the illness records of the fatigued athletes showed features consistent with a syndrome of re-activated EBV infection. Ninety per cent of the fatigued athletes were seropositive for previous EBV infection and 63% of this group were PCR positive for EBV DNA in saliva on one or more occasions before the probiotic treatment. Compared with the healthy control athletes, the fatigued athletes had more frequent and protracted episodes of upper respiratory tract symptoms, usually linked to periods of intense training. In a prospective study of elite swimmers over a 30 day period of intense training, EBV shedding was detected in saliva in two thirds of the subjects studied with physician verified symptoms of viral illness, including sore throat at times of EBV re-activation.[6] Clinical observation of the illness pattern in the presence of EBV shedding, together with negative evidence for alternative aetiologies, suggest that re-activation of EBV infection is a significant cause of recurrent sore throats and fatigue seen in some athletes after intense training.[6] Non-primary EBV infection has also been suggested as a cause of oropharyngeal symptoms in otherwise healthy young adults.[12] Re-activation of EBV, with the detection of EBV DNA in saliva, has been reported in other groups exposed to physical, psychological, and environmental stressors, including Antarctic winter expeditioners[13] and astronauts preparing for space flight.[14] The clustering of throat symptoms in athletes seropositive for EBV further supports the linkage.[6]

The fluctuating nature of virus shedding, the reasonable health of most affected individuals, the association of shedding with recognised stressors, and the known biology of EBV containment, all suggest that a subtle T cell defect in control mechanisms contributes to EBV re-activation and virus shedding. It is likely that models of virus shedding in serious disease or after cytotoxic therapy with profound suppression of host T cell function have limited value in understanding the pathogenesis of EBV re-activation in otherwise normal but stressed patients. In the latter, traditional assessments of T cell function are usually normal.

Changes in immune variables in athletes have been studied over the past two decades.[1 2] High intensity exercise is associated with transient changes in many measures of immune function, including a fall in salivary IgA,[1] and protracted training regimens can cause more persistent reductions in salivary IgA concentrations.[2 3] Both the incidence and timing of ''sore throats'' and EBV shedding show an inverse correlation with salivary IgA.[6] It has been proposed that salivary IgA monitoring can identify those at risk of developing recurrent upper respiratory ''sore throat'' symptoms.[1] The demonstration of a low level of secretion of IFNγ from CD4 positive T cells in fatigued athletes suffering recurrent and prolonged episodes of sore throats is likely to be relevant to the pathophysiology of re-activation EBV disease. The whole blood culture assay has been used to detect subtle but relevant defects in cytokine secretion.[11] The presence of reduced IFNγ secretion, with normal IL4 secretion, may reflect a skewed T cell response to dendritic cell signals.[15] The ''antigen processing cell–CD4 positive T cell'' unit, reliant as it is on finely tuned and complex cell to cell communications, would be an appropriate target for modulation and dysregulation by mediators influenced by stressors such as intense exercise. Salivary IgA concentrations are likely surrogate markers of protection, and the suppression of salivary IgA after intense exercise is itself a probable consequence of altered T cell function.

The *L acidophilus* isolate used in this study enhanced mucosal immunity in a murine model of *Candida albicans* stomatitis.[16] In this model the DBA/2 strain clears infection slowly compared with the BALB/c strain because of less efficient generation of cytokines and subsequent cytokine dependent effector mechanisms.[16] Defective cytokine secretion and cytokine dependent effector mechanisms were reversed by treatment with the *L acidophilus*, as a result of activation of the Peyer's patch dependent common mucosal

What is already known on this topic

- Intense training in elite athletes has been linked to a reduction in IgA concentration in saliva, and this, along with an increase in shedding of EBV, predicts more frequent episodes of upper respiratory tract illness
- Containment of EBV is thought to be mediated by T cell dependent mechanisms, not IgA

What this study adds

- Fatigued athletes showed significantly less secretion of IFNγ from blood CD4 positive T cells than healthy controls, the first evidence of a T cell defect
- A month of daily administration of *L acidophilus* significantly increased secretion of IFNγ from T cells in fatigued athletes to levels found in healthy athletes, and increased the concentration of IFNγ in saliva of control athletes

system.[16] The stimulation of IFNγ secretion in the fatigued athletes after *L acidophilus* administration resembles the situation in the DBA/2 model. A possible mechanism to explain the observed changes is the activation of antigen presenting cells to modulate a defective signal to the T cell receptor. Certain *L acidophilus* isolates can engage toll-like receptors on the surface of antigen presenting cells, which in turn affects the balance of signal to the T cell and the subsequent cytokine secretion pattern.[17] An increase in IFNγ secretion following polyclonal stimulation of blood lymphocytes in under-nourished children[18] and children with cow's milk allergy[19] given different probiotic preparations is consistent with the cytokine secretion patterns in this study of athletes. This study, however, is the first to identify an increase in IFNγ secretion from whole blood cells and an increase in IFNγ concentration in saliva in man, which suggest a possible link between probiotic use in man and the observed reduction of mucosal infection in animal models, where saliva concentrations of IFNγ have increased after ingestion of *L acidophilus*.[16] However, there is a need to document a significant reduction in symptoms in a larger and extended study, a conclusion also suggested by review of data presented in a study of an antiviral agent, Valtrex.[7]

This study shows for the first time reduced IFNγ secretion in fatigued athletes, a defect that was reversed after treatment with *L acidophilus*. The relation of these findings to other groups with chronic fatigue illnesses requires further study to clarify mechanisms and define therapeutic guidelines. It is important to extend the present studies to include clinical outcome measures to assist practitioners in developing management strategies for athletes presenting with fatigue, recurrent sore throats, and impaired athletic performance.

ACKNOWLEDGEMENTS

We acknowledge the generous participation of the athletes involved in the study.

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The study was funded in part by DSM Food Specialties Australia, who provided the *Lactobacillus acidophilus* (LAFTI®-10).

Competing interests: RC is a consultant for DSM (but received no payment for this study). AH is an employee of DSM. AC was funded by DSM. None of the other investigators had links with DSM or related companies.

Ethics: The study was approved by the human research ethics committee of the University of Newcastle, NSW, Australia, and the Hunter Area Research Ethics Committee, John Hunter Hospital, Newcastle, NSW, Australia, and all subjects gave informed and signed consent.

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