Lymphocyte responses to influenza and tetanus toxoid in vitro following intensive exercise and carbohydrate ingestion on consecutive days

Nicolette C. Bishop,1 Gary J. Walker,1 Lee A. Bowley,2 Kate F. Evans,2
Karen Molyneux,2 Fiona A. Wallace,3 and Alice C. Smith2
1School of Sport and Exercise Sciences, Loughborough University, Loughborough, Leicestershire; 2Department of Infection, Immunity and Inflammation, University of Leicester, and John Walls Renal Unit, Leicester General Hospital, Leicester; and 3GlaxoSmithKline Nutritional Healthcare Research and Development, Slough, Berkshire, United Kingdom

Submitted 11 January 2005; accepted in final form 24 May 2005

Bishop, Nicolette C., Gary J. Walker, Lee A. Bowley, Kate F. Evans, Karen Molyneux, Fiona A. Wallace, and Alice C. Smith. Lymphocyte responses to influenza and tetanus toxoid in vitro following intensive exercise and carbohydrate ingestion on consecutive days. J Appl Physiol 99: 1327–1335, 2005. First published May 26, 2005; doi:10.1152/japplphysiol.00038.2005.—The effect of carbohydrate (CHO) ingestion on antigen- (rather than mitogen-) stimulated T-cell responses to prolonged, intensive exercise may give a more realistic insight into the effect of CHO on T-cell functional capacity and subsequent infection risk. This study investigated the effect of CHO ingestion during prolonged, intensive exercise on influenza- and tetanus toxoid-stimulated T-cell cytokine mRNA expression and proliferation. Mitogen- [phytohemagglutinin (PHA)] stimulated proliferation was assessed for comparison. Responses were assessed following exercise on consecutive mornings to determine any carryover effect. Fifteen male games players performed two exercise trials in a double-blind, randomized, crossover design. Each trial comprised 90 min of intensive, intermittent running on consecutive mornings, with either CHO (6.4% wt/vol) or placebo (PLA) beverage ingestion before, during, and after each bout of exercise. Postexercise CD3+ cell counts were higher in PLA than CHO on both days (P < 0.05). Antigen-stimulated T-cell cytokine mRNA expression was unaffected by exercise or CHO ingestion. Before exercise on day 2, T-cell proliferative responses to PHA, influenza, and tetanus toxoid were higher in CHO than PLA by 99, 80, and 58%, respectively (P < 0.05 for PHA, P < 0.05 for influenza and tetanus toxoid). At 1 h postexercise on day 2, PHA-induced proliferation was 70% higher in CHO than PLA (P < 0.05), yet there were no differences between trials for antigen-induced proliferative responses. Therefore, mitogen-induced T-cell proliferation following strenuous exercise and CHO does not necessarily reflect responses to specific antigens and, consequently, may not provide a good model for the situation in vivo.

immune; antigen; T cell; cytokine

T lymphocytes play a pivotal role in the orchestration and regulation of the immune response to pathogens. As central elements of the acquired immune system, one important consequence of a defect in T-cell function is an increased incidence of viral infections (3). Several epidemiological studies suggest that athletes suffer from an increased incidence of viral infections, typically of the respiratory tract, following acute bouts of intense competitive exercise or periods of increased training volume (1, 13, 17). Intense exercise is associated with transient decreases in mitogen-induced proliferation of T cells (14, 15, 22), and it has been speculated that this may account for the apparent increased susceptibility of athletes to viral infections. However, lymphocyte responses are antigen specific, and assessment of cell responses to mitogens, such as the plant lectins phytohemagglutinin (PHA) and concanavalin A, in vitro does not necessarily provide a good model for the in vivo situation. In support of this, acute stress in rats has been recently shown to produce differing proliferative responses to antigenic and mitogenic stimulation (6). Mitogens activate a high percentage of circulating T cells (as well as other cell types, e.g., B cells), making this a rather nonspecific method that might result in unrealistically large alterations in T-cell function. Furthermore, responses to mitogen may lack the sensitivity to detect subtle but highly relevant alterations in the activity of the specific T-cell subpopulations involved in defense against common pathogens.

Recently, it has been reported that ingesting carbohydrate (CHO) at regular intervals during intensive exercise minimizes exercise-induced alterations in mitogen-stimulated T-cell function (8, 9). Whether this effect of CHO translates into enhanced host defense against common pathogens, such as respiratory pathogens, remains unanswered. Since T-cell responses following mitogen stimulation do not necessarily reflect the specific T-cell responses to specific antigens, the observation that CHO minimizes exercise-induced alterations in mitogen-stimulated T-cell proliferation does not necessarily reflect any protective effect of CHO on host T-cell function following prolonged, strenuous exercise. It is important to exercise caution when extrapolating any alterations in isolated T-cell responses to the situation in vivo and, therefore, altered susceptibility for infection. Nevertheless, it could be argued that determining the effect of regular CHO beverage ingestion on T-cell responses to antigenic (rather than mitogenic) stimulation, particularly responses to common respiratory pathogens such as influenza virus, gives a more realistic insight into the effect of CHO on T-cell functional capacity and subsequent infection risk.

Measurements of T-cell proliferation in response to mitogen (or antigen) give an indication of the overall magnitude of the resultant T-cell response to a stimulatory agent. When recognizing antigen, T cells are stimulated to express cytokine mRNA, and further investigation of the profile of the cytokines produced by T cells in response to an antigenic challenge gives an indication of the dominant T-cell type and response elicited, i.e., a type 1 or a type 2 response. Cytokines produced by type
1 T cells (IL-2 and IFN-γ) promote cytotoxic responses against virally infected cells; therefore, alterations in these responses may potentially offer an insight into the mechanism for the apparent increased susceptibility for infection experienced by athletes. Cytokines produced by type 2 T cells (e.g., IL-4 and IL-5) promote humoral immune responses, e.g., antibody production. These responses are important in defense against extracellular pathogens such as tetanus toxoid. Both the percentage of type 1 T cells and mitogen-stimulated T-cell production of IL-2 and IFN-γ have been shown to decrease in response to exercise (20, 21). However, the pathogen specificity of these responses was not investigated. Subtle changes in the magnitude of proliferative responses following strenuous exercise may have greater significance when considered in the light of the dominant T-cell type, since proliferating cells may prove to be promoting a less effective type of response to that specific pathogen.

Although assessment of T-cell function by stimulation with a variety of antigen types is routinely used in clinical practice, to the authors’ knowledge, there are no studies that have used this approach to assess in vitro T-cell function following exercise and CHO supplementation in humans. Therefore, the aim of this study was to investigate the effect of CHO [compared with placebo (PLA)] ingestion during prolonged, intensive, intermittent running on T-cell proliferative responses and cytokine mRNA expression to the antigen derived from the respiratory pathogen influenza and the antigen derived from the systemic pathogen tetanus toxoid. T-cell proliferative responses to PHA were also assessed for comparison. Because many athletes (particularly games players) train only once a day, the T-cell responses were assessed following intensive exercise on consecutive mornings to determine whether there was any “carryover” effect from the previous day’s activity and the relative additive effect of consecutive days of exercise.

METHODS

Subjects. Fifteen male regular games players [mean ± SE: age 22 ± 0 yr; body mass 76.3 ± 1.7 kg; maximum O2 uptake (VO2 max) 54.8 ± 0.6 ml·kg⁻¹·min⁻¹] volunteered to participate in the study. All subjects were informed about the rationale for the study and the nature of the exercise tests to be performed before providing written, informed consent. The Ethics Committee of Loughborough University approved the protocol. Subjects were required to complete a comprehensive health-screening questionnaire before each exercise test and did not report any symptoms of infection and had not taken any medication in the 6 wk before the study, nor were they currently on medication. None of the subjects had been previously immunized against any strain of influenza virus, nor had they experienced influenza in the previous 2 yr. All of the subjects had received the tetanus “booster” vaccination at age 16 yr as part of the immunization program provided by the UK Department of Health, but none had required immunization since that time.

Overall experimental design. Subjects performed two exercise trials in a randomized, crossover design. Each trial comprised 90 min of high-intensity intermittent shuttle running on two consecutive mornings. For one trial, subjects ingested CHO beverages (6.4% wt/vol glucose and maltodextrin) before, during, and after both bouts of 90-min intermittent running (CHO trial). For the other trial, subjects ingested a water-based, artificially sweetened PLA drink before, during, and after both bouts of 90-min intermittent running (PLA trial). Each drink was identical in flavor (orange) and appearance, and the subjects were unaware of the content of the drinks in each trial.

Preliminary testing. Approximately 10 days before main exercise trials, each subject’s VO2 max was estimated by means of performing a progressive shuttle run test (18). From this estimate, running speeds corresponding to 55 and 95% VO2 max were calculated from the tables for predicted VO2 max values (18). Subjects then returned to the laboratory 1 wk before the first main trial to perform 90 min of intermittent shuttle running to familiarize themselves with the required running speeds and experimental procedures: following a 10-min standardized warm-up, subjects completed six 15-min periods of intermittent shuttle running consisting of ~10 cycles of maximal sprinting, running, and walking (Fig. 1). The subjects were required to run between two lines, 20 m apart, at various speeds that related to the previously estimated VO2 max values. The running and walking speeds during each of the 20 m of the test were dictated by an audio signal from a computer by using software developed for this purpose. The 15-min bouts of running were separated by 3-min rest periods. Samples of expired air were collected during the second, fourth, and sixth 15-min block of exercise using Douglas bags to ensure that the subjects were exercising at the required intensity. If the subject was exercising at <70% VO2 max or in excess of 80% VO2 max, running speeds were adjusted accordingly. Heart rates were monitored throughout this familiarization trial using short-range radio telemetry (Polar Beat, Polar Electro, Oy, Finland).

Experimental trial procedures. During the 48 h preceding each experimental trial, subjects were asked to refrain from training and required to record their food intake in food diaries in an effort to standardize their nutritional status. They were required to eat the same foods during the 48 h preceding the second experimental trial. Energy intake and dietary macronutrient composition were subsequently analyzed using the “CompEat” dietary analysis computer software package (version 5, Nutrition Systems, Grantham, UK).

Subjects reported to the laboratory at 8:00 AM for each exercise trial following an overnight fast of at least 10 h and were randomly assigned to either the CHO or PLA trial. Subjects were then required to empty their bladder before body mass (in shorts only) was recorded. Subjects then sat quietly for 10 min before an initial resting blood sample (40 ml) was obtained by venipuncture from an antecubital vein. The 10-min standardized warm-up was performed, and subjects then consumed 5 ml/kg body mass of either CHO solution (CHO trial) or an artificially sweetened PLA solution (PLA trial).

Subjects began 90 min of intermittent shuttle running within 5 min of consuming the prescribed beverage. During the 3-min rest periods that separated each of the six 15-min bouts of running, subjects consumed a further 2 ml/kg body mass of the prescribed solution. Heart rate was measured continuously throughout the exercise using short-range radio telemetry (Polar Beat, Polar Electro) and measurements of O2 uptake and CO2 production were made during the second, fourth, and sixth block of exercise by means of expired air collection in Douglas bags. A paramagnetic oxygen analyzer (Servomex 1420B, Crowborough, UK) and an infrared carbon dioxide analyzer (Servomex 1415B) were used along with a dry-gas meter (Harvard Apparatus, Edenbridge, UK) for determination of O2 uptake and CO2 production. The mean exercise intensity (%VO2 max) for the full 90 min of exercise was calculated from these measurements, excluding the 3-min rest periods. Further blood samples (each 40 ml) were taken with the subject seated immediately postexercise and at 1 h postexercise. Immediately after the postexercise blood sample had been obtained, body mass (in shorts only) was again recorded. On both trials, subjects then consumed 5 ml/kg body mass of the prescribed beverage. No other fluid or food intake was allowed until after the 1-h postexercise blood sample had been obtained. For all trials, the laboratory temperature was between 15 and 18°C, and relative humidity was 56 ± 3%.

After performing the first 90-min bout of intermittent running of each exercise trial, subjects refrained from further strenuous physical activity for the remainder of that day and recorded their dietary intake. They again fasted overnight for a period of at least 10 h before
returning to the laboratory at 8:00 AM on the following morning to complete the second bout of intermittent running of that exercise trial. Following an interval of at least 7 days, subjects returned to the laboratory to perform the second exercise trial with either CHO or PLA ingestion before, during, and after both bouts of the high-intensity intermittent running.

**Total and differential leukocyte counts and plasma volume.** Blood samples were collected into three separate monovette tubes (evacuated blood collection tubes, Sarstedt, Leicester, UK): one containing K3-EDTA (1.6 mg EDTA/ml blood) and two containing lithium heparin (1.5 IU heparin/ml blood). Blood taken into the K3-EDTA monovette (2.7 ml) was used for hematological analysis, including hemoglobin, hematocrit, and total and differential leukocyte counts using an automated cell-counter (Ac/T 5diff hematology analyzer, Beckman Coulter, High Wycombe, UK). All cell counts were corrected for plasma volume changes relative to the first blood sample for each bout of exercise; these were estimated from the hemoglobin and hematocrit values, according to Dill and Costill (2).

**Assessment of lymphocyte subpopulations by flow cytometry.** Expression of CD3, CD4, CD8, CD19, and CD56 by peripheral blood cells was determined by flow cytometry, using Pharmingen monoclonal and isotype control antibodies purchased from Becton Dickinson Biosciences (Oxford, UK). Aliquots of whole heparinized whole blood were labeled with cocktails of the following monoclonal antibodies against human lymphocyte cell surface markers: FITC-conjugated anti-CD3, phycoerythrin- (PE) conjugated anti-CD4 and anti-CD19, and R-phycocerythrin-Cy5- (PE-Cy5) conjugated anti-CD8 and anti-CD56. Labeling was carried out on ice, and the cells were subsequently washed once in ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin and 2 mM EDTA (PBS/BSA/EDTA), after which the erythrocytes were lysed with FACS Lyse (Becton Dickinson), and the leukocytes resuspended in PBS-BSA-EDTA. Three-color flow cytometric analysis was carried out by using a FACScan flow cytometer with CellQuest analysis software (Becton Dickinson). Side-scatter vs. forward-scatter plots were used to gate on the lymphocyte population by morphology, and 30,000 lymphocyte events were acquired per analysis. Cells incubated with appropriate fluorescent-labeled isotype control antibodies were used to define the thresholds of positive staining for each marker. The percentages of T and B cells were derived from quadrant analysis of FL1 (CD3 FITC) and FL2 (CD19 PE), and natural killer (NK) cells from FL1 (CD3 FITC) and FL3 (CD56 PE-Cy5), defining the NK population as CD3-CD56−. To derive the percentage of T cells expressing CD4 and CD8, the FL1 CD3+ population was subjected to quadrant analysis of FL2 (CD4 PE) vs. FL3 (CD8 PE-Cy5) on dot plots.

**In vitro stimulation of antigen-specific T cells.** Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood by density gradient centrifugation. The cells were washed in sterile isotonic salt solution and resuspended in culture medium (RPMI-1640 containing 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM HEPES, and 24 mM Na2CO3). The PBMCs were counted, and the cell suspensions were diluted to achieve a final concentration of 10⁶ T cells/ml, calculated from the percentage CD3+ T cells for each sample obtained from the flow cytometry described above.

Aliquots of cells (200 µl) were cultured in flat-bottomed 96-well microtiter plates in medium alone (unstimulated baseline control) or with 5 µg/ml PHA (Sigma, Poole, Dorset, UK), influenza vaccine at 1:1,000 final dilution (Influvacc 2001/2, Solvay Healthcare, Southampton, UK), or tetanus vaccine at 1:1,000 final dilution (Adsorbed Tetanus Vaccine, Pasteur Merieux MSD, Maidenhead, Berks, UK). Preliminary titrations of both antigens and PHA were performed in an initial pilot study of a number of young, healthy subjects. The doses used in this study were those in the middle of the optimal range for both antigens and PHA for the subjects of this pilot study that gave an equated amount of proliferation. Replicate wells were set up for each condition, and the cells were incubated at 37°C in a humid 5% CO₂ atmosphere. These stimulated cells were used for assessment of T-cell mRNA expression and T-cell proliferation, as described below.
Table 1. Primer and probe sequences used in the real-time PCR analysis for the expression of mRNA for Cβ, IFN-γ, IL-2, IL-4, and IL-5 by antigen-stimulated T cells

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Oligonucleotide Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cβ</td>
<td>Forward primer: tcacgttctccagggcttcg</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: aagagcggccagcatggag</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward primer: aagagacagagtctagttcga</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: tcaccctactctttgaggt</td>
</tr>
<tr>
<td>Taqman probe</td>
<td>Tgaccttgcaagacacctggtat</td>
</tr>
<tr>
<td>IL-2</td>
<td>Forward primer: aactccacagggtctccca</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: gcacccctccgaaggcagttg</td>
</tr>
<tr>
<td>Taqman probe</td>
<td>Tgaccccaagaaacggcggcag</td>
</tr>
<tr>
<td>IL-4</td>
<td>Forward primer: tctatttttcctcctgattcag</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: tggaggatgagaccctatata</td>
</tr>
<tr>
<td>Taqman probe</td>
<td>Tgccctacactttgctacctcaagttcag</td>
</tr>
<tr>
<td>IL-5</td>
<td>Forward primer: cgttctcagacgaccttgagat</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: accaatgcaccttggaagat</td>
</tr>
<tr>
<td>Taqman probe</td>
<td>Tgcctagctgatgtaacctcctccc</td>
</tr>
</tbody>
</table>

**Cβ, T-cell receptor β-chain.**

### T-cell cytokine mRNA expression

After 48 h in culture, ~2 × 10^6 PBMCs were harvested for each culture condition. The cells were pelleted, the supernatant removed, and the cells washed once in 1 ml sterile, RNase-free PBS. RNA was extracted from the cells by using a commercial kit (RNAqueous-4PCR, Ambion Europe, Huntingdon, UK), following the manufacturer’s instructions. Briefly, the cells were lysed in the presence of guanidium thiocyanate, and the lysate passed through a silica-based filter to immobilize the RNA during a series of washes. RNA was eluted from the filter and treated with DNase to remove traces of contaminating DNA. Finally, the DNase was inactivated and cDNA was synthesized from the RNA samples with avian myeloblastosis virus reverse transcriptase and random primers using a commercial kit (Reverse Transcription System, Promega, Southampton, UK). cDNA samples were stored at −80°C until PCR analysis.

Real-time PCR was used to analyze the expression of mRNA for IFN-γ, IL-2, IL-4, and IL-5 by antigen-stimulated T cells. In addition, expression of mRNA for the constant region of the T-cell receptor β-chain (Cβ) was also assessed to correct for the varying proportions of αβ-T cells in the PBMC samples. Primers and probes were designed in-house (sequences shown in Table 1). Primers were custom synthesized by Invitrogen (Paisley, UK), and 5′-FAM/3′-TAMRA Taqman probes by Sigma Genosys (Cambridge, UK). PCRs were carried out in duplicate 50-μl volumes containing 2 μl cDNA, 1 μl forward and reverse primers (10 μM), 2 μl Taqman probe (10 μM), and 1.25 units Taq polymerase in a buffer containing each of the four dNTPs at 0.2 mM final concentration. Negative controls containing water instead of cDNA were set up in each run. Reactions were performed on a Stratagene MX4000 instrument starting with 10 min at 95°C, followed by 50 cycles of 30 s at 95°C, 60 s at 59°C, and 30 s at 72°C, setting a standard threshold fluorescence of 0.1 for all experiments.

Semiquantification of the PCRs was achieved by including four serial dilutions of a standard PBMC-derived cDNA sample in each PCR run. The standard cDNA was assigned an arbitrary unit (AU) value for each cDNA species assayed, and standard curves were constructed by plotting log_{10} AU against threshold cycle number for each standard dilution. For all cDNA species analyzed, the relationship between sample dilution and threshold cycle number was linear over at least a 1000-fold dilution range. The relative cDNA content of the test samples was then read from the curve in AU, and cytokine values were corrected for Cβ expression in each sample. In this way, the relative mRNA expression of a given cytokine could be compared between samples, although the mRNA expression of the different cytokines could not be evaluated.

**T-cell proliferation assays.** The proliferation of T cells responding to PHA, influenza vaccine, and tetanus toxoid vaccine in vitro was measured by the incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) after 7 days in the culture conditions described above. On the 7th day, the cells were pulsed with 10 μM BrdU and returned to the incubator for a further 24 h. BrdU incorporation was measured by using a commercially available ELISA system (Cell Proliferation ELISA, Roche Diagnostics, Mannheim, Germany), following the manufacturer’s instructions. Briefly, the microtiter plates were centrifuged at 300 g for 10 min to immobilize the cells on the bottom of the wells, and the supernatant medium was removed. The wells were dried for 1 h at 60°C, the cells were fixed, and the DNA was denatured by adding 200 μl/well fixative-denaturation solution for 30 min at room temperature. After the supernatant was decanted, 100 μl-well 100-μl peroxidase-conjugated anti-BrdU solution^1 was added and incubated for 90 min at room temperature, and the wells were washed four times in the wash buffer provided in the kit. Finally, 100 μl/well chromogenic substrate was added for 10 min before stopping with 25 μl-well 100-μl M H_2SO_4. The absorbances of the wells were read at 450 nm (A_{450}) and the reference wavelength of 710 nm (A_{710}). The absorbance value at the reference wavelength was subtracted from that at 450 nm for each well, and the means of duplicate wells were calculated for each sample. The proliferation indexes (PIs) of the cultures stimulated by PHA, influenza, and tetanus toxoid, respectively, were derived from the formula:

\[
\text{PI} = \frac{A_{450} - A_{710}}{A_{450} - A_{710}} \times 100
\]

**Plasma cortisol, glucose, and lactate.** Heparinized plasma was obtained from blood collected into a further lithium heparin monovette (7.5 ml). This was spun at 1,500 g for 10 min in a refrigerated centrifuge (4°C) within 5 min of sampling. The plasma obtained was immediately stored at −80°C for later analysis. Plasma cortisol and glucose concentrations were determined using ELISA (EIA-1887, IDS, Boldon, UK) and colorimetric (GOD-PAP method, Randox Laboratories, Antrim, UK) methods, respectively. Plasma lactate was determined in deproteinized plasma using a standard spectrophotometric method, as described by Fink and Costill (4). For each assay, all samples were analyzed on the same day. The intra-assay coefficient of variation was 1.8% for cortisol, 1.6% for glucose, and 2.8% for lactate.

**Statistical analysis.** Data in the text, Tables 2–4, and Figs. 2–6 are presented as mean ± SE. The data were examined using a two-factor...
Total and differential leukocyte counts in response to the exercise protocol with carbohydrate and placebo beverage ingestion

Table 3. Plasma cortisol and lactate response to the exercise protocol with carbohydrate and placebo beverage ingestion

![Table 2. Plasma cortisol and lactate response to the exercise protocol with carbohydrate and placebo beverage ingestion](http://jap.physiology.org/)

Table 3. Total and differential leukocyte counts in response to the exercise protocol with carbohydrate and placebo beverage ingestion

![Table 3. Total and differential leukocyte counts in response to the exercise protocol with carbohydrate and placebo beverage ingestion](http://jap.physiology.org/)
Numbers of circulating lymphocyte subsets are given in Table 4. An interaction effect was observed for numbers of CD3+ cells [F(5,70) = 4.4, P = 0.02], with numbers lower in CHO than PLA at postexercise on day 1 and 1 h postexercise on day 2. In the PLA trial, the circulating CD3+ count at preexercise on day 2 was 18% lower than at preexercise on day 1 but did not differ in CHO. No other significant interaction effects were observed for numbers of CD4+ T cells, CD8+ T cells, B cells, or NK cells. However, there was an effect of exercise on CD8+ T cells, with counts lower than preexercise values on day 1 at preexercise on day 2 [main effect for time, F(5,70) = 15.3, P < 0.001].

Quantifiable expression of Cβ, IFN-γ, IL-2, IL-4, and IL-5 was detected in the majority of the cDNA samples prepared from antigen-stimulated PBMCs. Samples with undetectable Cβ levels were excluded from the analysis, as this indicates an insufficient amount of viable cDNA; this reduced the n value for these analyses to 9. The results obtained from viable samples repeated on different occasions were extremely reproducible for Cβ and all of the cytokines (coefficients of variation <2% for all PCRs throughout the study). However, there was a high degree of variability in the cytokine expression levels of the different samples, and no consistent expression patterns or trends were discernable. No significant interaction effects or main effects for time or trial were observed for influenza-stimulated T-cell expression of the Type 1 cytokines IL-2 (Fig. 3) and IFN-γ (data not shown) or the Type 2 cytokines IL-4 and IL-5 (data not shown). Similarly, no significant interaction effects or main effects for time or trial were observed for tetanus toxoid-stimulated T-cell expression of IL-2, IFN-γ, IL-4, or IL-5 (data not shown).

In the proliferation assays, good proliferative responses to PHA, influenza, and tetanus toxoid were observed, with BrdU incorporation increased at least threefold in stimulated cells compared with parallel unstimulated cultures. Significant interaction effects were observed for both mitogen- [PHA: F(5,70) = 4.3, P = 0.002] and antigen- [influenza: F(5,70) = 2.9, P = 0.018; tetanus toxoid: F(5,70) = 3.0, P = 0.017] stimulated proliferative responses (see Figs. 4–6). At preexercise on day 2, proliferative responses to all three stimulants were higher on CHO than PLA (by 99, 80, and 58% for PHA, influenza, and tetanus toxoid, respectively). In the PLA trial at this time, proliferative responses to each stimulant were ~40%

---

**Table 4. Lymphocyte subsets in response to the exercise protocol with carbohydrate and placebo beverage ingestion**

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th></th>
<th>Day 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post 1 h</td>
<td>Pre</td>
<td>Post 1 h</td>
</tr>
<tr>
<td>CD3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>1.45±0.13</td>
<td>1.44±0.11*</td>
<td>1.03±0.07</td>
<td>1.33±0.09</td>
</tr>
<tr>
<td>PLA</td>
<td>1.46±0.11</td>
<td>1.70±0.15</td>
<td>1.08±0.07</td>
<td>1.20±0.10†</td>
</tr>
<tr>
<td>CD3+ CD4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>0.73±0.07</td>
<td>0.83±0.09</td>
<td>0.61±0.03</td>
<td>0.68±0.06</td>
</tr>
<tr>
<td>PLA</td>
<td>0.74±0.05</td>
<td>0.77±0.06</td>
<td>0.60±0.04</td>
<td>0.67±0.07</td>
</tr>
<tr>
<td>CD3+ CD8+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>0.55±0.06</td>
<td>0.71±0.08</td>
<td>0.38±0.05</td>
<td>0.48±0.05</td>
</tr>
<tr>
<td>PLA</td>
<td>0.57±0.06</td>
<td>0.71±0.09</td>
<td>0.37±0.05</td>
<td>0.43±0.05</td>
</tr>
<tr>
<td>CD19+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>0.21±0.03</td>
<td>0.19±0.02</td>
<td>0.20±0.02</td>
<td>0.20±0.03</td>
</tr>
<tr>
<td>PLA</td>
<td>0.20±0.02</td>
<td>0.22±0.02</td>
<td>0.20±0.02</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>CD3+ CD56+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>0.21±0.04</td>
<td>0.30±0.05</td>
<td>0.13±0.02</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>PLA</td>
<td>0.21±0.03</td>
<td>0.34±0.06</td>
<td>0.13±0.02</td>
<td>0.15±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 15. *Significantly lower on CHO compared with PLA, P < 0.05; †significantly lower than at the same time on day 1, P < 0.05; ‡main effect for time with preexercise day 2 lower than preexercise day 1, P < 0.001.
lower than before exercise on \textit{day 1}. At 1 h postexercise on \textit{day 2}, PHA-stimulated proliferative responses were 70% higher on CHO than PLA. No differences were found in antigen-stimulated responses at this time.

\textbf{DISCUSSION}

The main findings of this study were that prolonged, intensive exercise on consecutive mornings was associated with reduced antigen-stimulated proliferative responses before exercise on \textit{day 2} compared with \textit{day 1}. These responses occurred despite no apparent effect of exercise on antigen-stimulated T-cell cytokine mRNA expression. In addition, CHO beverage ingestion attenuated the decrease in antigen-stimulated proliferative responses before exercise on \textit{day 2} but had no effect on T-cell cytokine mRNA expression. Furthermore, CHO ingestion was associated with greater T-cell proliferative responses after exercise on \textit{day 2} following mitogen stimulation only.

To the authors’ knowledge, this is the first study to have assessed the acute effect of prolonged, intensive exercise on in vitro antigen-stimulated T-cell cytokine expression and proliferative responses in humans. It is also the first study to have assessed the role of CHO beverage ingestion on these antigen-specific responses. Several previous studies of acute, prolonged, strenuous exercise report a decrease in T-cell proliferative responses to a variety of mitogens, including the plant lectins PHA and concanavalin A (9, 12). It has been speculated that this may account for the observed higher incidence of respiratory infection in athletes involved in heavy schedules of training and competition. However, mitogens activate other cell types, including B cells and NK cells, in addition to a high percentage of circulating T cells (19). Therefore, mitogen stimulation may result in unrealistically large alterations in proliferative responses that may not accurately reflect specific responses to antigens of relevance to this population, such as influenza. The findings of the present study appear to support this; the pattern of change for influenza- and tetanus toxoid-stimulated proliferative responses was similar, yet, following exercise on \textit{day 2}, CHO ingestion was found to influence proliferative responses to PHA only. It could be argued that differences in the magnitude of the proliferation response to PHA and antigen stimulation could be related to the dose of mitogen and antigen used. However, in the present study, this is unlikely to be the major influencing factor in the responses observed, because the concentration of PHA and each antigen used was designed to give an equated amount of proliferation, as determined from the results of an initial pilot study of resting young, healthy subjects. Caution should always be taken when extrapolating the findings of any in vitro measure, be it antigen or mitogen stimulated, to the in vivo situation. Nevertheless, these findings may highlight a need for some caution when extrapolating mitogen-stimulated T-cell responses to those of common pathogens and to a subsequent risk of infection.

In an attempt to gain an indication of the effect of exercise (and CHO) on the dominant T-cell response elicited, we also investigated T-cell expression of Type 1 and Type 2 cytokines.
using antigens that would predominantly stimulate a Type 1 (influenza) or a Type 2 (tetanus toxoid) T-cell response. Assessment of T-cell subset activation is challenging. In our study, secreted cytokine protein levels in culture supernatants were insufficient for measurement, and, therefore, we evaluated the relative Type 1 and Type 2 T-cell responses by real-time PCR for cytokine mRNA expression. One possible reason for the observed higher incidence of infections in elite athletes may be a change in the bias of the T-cell cytokine response for a particular antigen, in addition to a reduction in T-cell proliferation. Prolonged, strenuous exercise is associated with a decrease in the percentage of Type 1 T cells in the circulation (21), and shorter intensive exercise is associated with decreases in mitogen-stimulated T-cell release of IL-2 and IFN-γ (20). Therefore, we hypothesized that any reductions in antigen-stimulated proliferative responses would be associated with a decrease in Type 1 T-cell cytokine mRNA expression and a dominant Type 2 T-cell response. However, our findings do not support this: neither exercise (nor CHO ingestion) affected Type 1 or Type 2 T-cell cytokine expression, despite exercise-induced reductions in antigen-stimulated cellular proliferation, although it must be borne in mind that mRNA expression does not necessarily relate directly to synthesis and secretion of functional cytokine protein. Previous studies that have assessed T-cell cytokine release in response to exercise have used phorbol myristate acetate as a stimulating agent (20, 21); therefore, it could be speculated that our findings are reflecting a specific response to specific antigens. Unfortunately, we cannot confirm this since mitogen-stimulated T-cell cytokine mRNA expression was not assessed in this study.

On the other hand, there may be an alternative explanation for these observations: Green and Rowbottom (7) report that the observed reduction in mitogen-stimulated T-cell proliferation following intensive exercise is not due to a decrease in mitosis but rather an increase in the rate of cell death in culture; i.e., there is a reduction in the number of cells that are able to respond. In the present study, cellular proliferation and cytokine mRNA expression were assessed from cultures that contained a T-cell concentration of 10⁶/ml for all samples collected. Therefore, any exercise-induced increase in the rate of cell death would reduce the number of responsive cells and could be expected to account for the observed decrease in antigen-stimulated proliferation before and after exercise on day 2 in the PLA trial. However, any alteration in the number of responsive cells in response to exercise would not be reflected in the observations for T-cell cytokine mRNA expression. This is reported as a ratio relative to the expression of mRNA for the T-cell cytokine-specific gene Cβ, which corrects cytokine mRNA expression for the relative presence of total viable T-cell mRNA. In this way, if there is a decrease in the expression of mRNA for a particular T-cell cytokine, the ratio will decrease, and vice versa. If increased rates of cell death are responsible for the alterations in proliferative responses observed here, they would simply not be reflected in the observations for T-cell cytokine mRNA expression, since dead cells will not express patent mRNA for either Cβ or the T-cell cytokines; i.e., the ratio would be unaffected. Nevertheless, an increase in the rate of cell death could still account for a decreased percentage of circulating Type 1 T cells following prolonged, intensive exercise (21).

Altered rates of cell death may also account for the finding of higher antigen- and mitogen-stimulated proliferative responses in CHO than in PLA before exercise on day 2, since Green et al. (8) found that regular CHO ingestion during exercise was associated with reduced rates of cell death within mitogen-stimulated cell cultures. Previously, the effect of CHO ingestion in preventing the reduction of PHA-stimulated T-cell proliferative responses to prolonged, intensive exercise has been associated with a concurrent attenuation of the cortisol response (9). However, the findings of our study and others (8) suggest that the role of cortisol is perhaps not as crucial as was previously thought, since the effects of CHO on antigen- (and mitogen-) stimulated T-cell proliferation occurred without any influence of CHO on plasma cortisol concentration. Nevertheless, the higher proliferative responses following antigen stimulation reported here may reflect a protective effect of CHO for subsequent infection risk, since a recent study in horses reported decreased T-cell proliferative responses to equine influenza virus following 5 days of strenuous exercise (5). These horses also demonstrated increased susceptibility to influenza following a later challenge exposure to the same strain of the virus.

The effect of exercise performed on consecutive mornings was assessed, since many athletes, particularly games players, typically train only once a day. Recently, playing a game of soccer on consecutive days reduced the expression of lymphocyte adhesion and signaling molecules 6 h after the second game (11). However, this study did not take any measurements after the first game or immediately before the second game, making it difficult to assess whether there was any carryover effect from the first game. In PLA in the present study, influenza- and tetanus toxoid-stimulated proliferative responses were lower before exercise on day 2 compared with the same time on day 1. Furthermore, our findings suggest that CHO ingestion may help to diminish this effect. These responses cannot be accounted for by alterations in the numbers of circulating CD3⁺ cells, since proliferation was assessed in a constant concentration of T cells for all samples.

In an attempt to overcome previous concerns (7) regarding the assessment of exercise-induced changes in T-cell proliferation using cultures containing a constant number of PBMCs, we assessed antigenic and mitogenic proliferative responses in cultures of PBMCs containing a constant number of T cells. This aimed to overcome potential concerns arising from the interpretation of post hoc mathematical adjustments to proliferation data from mixed-cell cultures as changes in T-cell function (7). However, this methodology itself gives rise to a number of limitations that must be acknowledged when interpreting these findings. Although the cultures contained constant numbers of T cells, there would have been alterations in other cell populations (e.g., monocytes and B cells) present in the cultures that could contribute to the overall proliferative response observed. Whereas PHA is a potent stimulant of T cells because it binds mainly to CD3 (19), we were not able to determine which cells are actively proliferating in response to the specific antigens influenza and tetanus toxoid. Therefore, we cannot simply assume that the antigenic proliferative responses observed are solely reflecting alterations in T-cell activity. Furthermore, exercise-induced alterations in monocyte function could influence the magnitude of any subsequent antigen-specific proliferative response, since monocytes can...
directly alter cellular proliferation. For example, nitric oxide and PGE2 are known to suppress proliferation, and increases in monocyte and macrophage release of these substances have been shown after exercise (10, 16). These factors could also contribute to the effects of CHO and exercise on PHA-stimulated proliferative responses, yet they may not be detectable in this data because of the greater magnitude of the proliferative response to mitogen (i.e., any monocyte-mediated effect was too subtle to be detected within a larger mitogen-stimulated response, but it may be more apparent within the smaller overall response to antigenic stimulation).

In summary, the findings of this study suggest that CHO ingestion during exercise attenuates the reduction in cellular proliferative responses to influenza and tetanus toxoid in a manner that appears to be independent of changes in plasma cortisol concentration and T-cell cytokine mRNA expression. In addition, these findings indicate that T-cell proliferative responses to mitogen following exercise and CHO ingestion do not necessarily reflect proliferative responses to specific antigens. Therefore, assessment of proliferative responses to specific antigens (rather than mitogen) in vitro following exercise may provide a more realistic model for the situation in vivo and subsequent infection risk.

ACKNOWLEDGMENTS

We thank Dr. Giles Walters for kindly providing us with TCR Cβ primer and probe sequences.

GRANTS

This study was supported by a grant from GlaxoSmithKline Nutritional Healthcare.

REFERENCES