Distribution and mitogen response of peripheral blood lymphocytes after exertional heat injury

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DuBose, David A., C. Bruce Wenger, Scott D. Flinn, Thomas A. Judy, Alexandre I. Dubovtsev, and David H. Morehouse. Distribution and mitogen response of peripheral blood lymphocytes after exertional heat injury. J Appl Physiol 95: 2381–2389, 2003.—To determine whether immune disturbances during exertional heat injury (EHI) could be distinguished from those due to exercise (E), peripheral lymphocyte subset distributions and phytohemagglutinin-stimulated CD69 mitogen responses as discriminated by flow cytometry were studied in military recruits [18.7 ± 0.3 (SE) yr old] training in warm weather. An E group (3 men and 3 women) ran 1.75–2 miles. During similar E, 11 recruits (10 men and 1 woman) presented with suspected EHI. EHI (40.4 ± 0.3°C) vs. E (38.6 ± 0.2°C) body temperature was significantly elevated (P < 0.05). Heat illness was largely classified as EHI, not heatstroke, because central nervous system manifestations were generally mild. Blood was collected at E completion or EHI onset (0 h) and 2 and 24 h later. At 0 h (EHI vs. E), suppressor, natural killer, and total lymphocyte counts were significantly elevated, helper and B lymphocyte counts remained similar, and the helper-to-suppressor ratio was significantly depressed. By 2 h, immune cell dynamics between groups were similar. From 0 to 24 h, T lymphocyte subsets revealed significantly reduced phytohemagglutinin responses (percent CD69 and mean CD69 fluorescent intensity) in EHI vs. E. Thus immune cell dynamics with EHI were distinguishable from E. Because heat stress as reported in exercise or heatstroke is associated with similar immune cell disturbances, these findings in EHI contributed to the suggestion that heat stress of varying severity shares a common pathophysiological process influencing the immune system.

leukocytes; lymphocyte subsets; hyperthermia; heat stress; heatstroke; flow cytometry; exercise; phytohemagglutinin

HEATSTROKE IS CHARACTERIZED by hyperthermia and neurological abnormalities such as delirium, seizures, stupor, or coma (3, 10, 33). Heatstroke patients generally have rectal temperatures (T rek) >41.1°C (10). Heatstroke is also characterized by immune dysfunction, including disturbances in the distribution of peripheral lymphocyte subsets (2), as well as changes in other immune system elements (12, 13, 24). Historically, heat illness with systemic manifestations has been divided into heatstroke and heat exhaustion, a disorder characterized chiefly by disturbances of cardiovascular homeostasis. Some authors (6, 15, 19), however, have proposed that heat exhaustion and heatstroke should be thought of as parts of a continuum, rather than as distinct disorders. Moreover, many cases of heat illness occurring in association with strenuous exercise do not have significant neurological abnormalities but, rather, show signs of tissue injury or organ dysfunction beyond disturbances of cardiovascular homeostasis. For such cases, the choice between heat exhaustion and heatstroke seems inappropriate, and the term “exertional heat injury” (EHI) has come to apply to a syndrome involving hyperthermia with evidence of organ system dysfunction that is less severe than heatstroke. In this study, EHI refers to a syndrome occurring in association with strenuous exercise characterized by hyperthermia with evidence of organ system dysfunction, but without significant neurological disturbances or impairment of mental status, as noted in heatstroke.

Although in the absence of heat illness, exercise alone alters lymphocyte subset distribution and mitogen responses (7, 8, 14, 21–27, 29–31, 35, 36), it is hypothesized that immune dysfunction associated with even the mild forms of heat illness, such as EHI, can be distinguished from the effects of exercise. Previous reports of immune dysfunction in heatstroke (1–5, 12, 13, 32), the most severe form of heat illness, have limitations relative to their accounting for the effects of exercise. The present study controlled for exercise by comparing immune system parameters of EHI patients with subjects who conducted a similar type and length of exercise but did not suffer heat illness. Such a study may provide insight to the influences of exercise and hyperthermia on immune dysfunction.

MATERIALS AND METHODS

Of 130 subjects enrolled in a larger clinical investigation of heat tolerance and exertional heat illness as controls or the 21 experiencing heat illness, this report describes findings on 6 controls and 11 heat illness cases. This subset was selected specifically to closely match the type of physical activity

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associated with the onset of heat illness to that of the controls.

Two groups of subjects were studied: an exercise control group (E) and a group of patients diagnosed largely with EHI. Members of the E group were typical US Marine Corps recruits (3 men and 3 women) undergoing basic training at the US Marine Corps Recruit Depot, Parris Island, SC, during September 2000. Prospective subjects were briefed on the aims of the study and its expected risks and discomforts. At that time, subjects gave their informed consent. On the evening before the run, each subject swallowed an ingestible telemetric temperature sensor (CorTemp, HTI Technologies, St. Petersburg, FL) to ensure that it would be past the stomach and insensible to swallowed hot or cold liquids by the time of the run. During the run, the signal from the sensor was converted to a temperature and recorded every 30 s by a receiver-recorder (Body Core Temperature Monitors, version 3 or 4, FitSense, Southborough, MA) belted at the subject's waist. On the following morning, each E subject provided a 3-ml heparinized blood sample before the run (pre-E). These subjects then performed a 1.75- to 2-mile running exercise as part of their normal training program. Blood was also collected just at the end of E (time 0) and ~2 and 24 h after E.

Recruits who arrived at Parris Island during the months of May–September 2000 were briefed on the EHI aspect of the study during in-processing and asked to volunteer to be tested should they experience a heat illness during basic training. Those who volunteered were entered into a computerized database. Heat illness was generally manifested by inability to continue in a training exercise event or by collapse after the end of an event. The standard management of a suspected heat illness patient in the field included measurement of 

\[ T_{es} \]

and wetting down the patient with cold water. The patient was then transported to the Branch Medical Clinic (BMC) at Parris Island, where 

\[ T_{es} \]

was again measured. Time between onset of an episode of heat illness and arrival at the BMC was typically 10–15 min. On arrival, patients were stripped to their underwear and cooled by covering in sheets wetted with ice water followed by a tepid shower. During cooling, vital signs and assessment of mental status were recorded every few minutes. When a heat illness patient arrived at the BMC and before collection of blood for the study, the computerized database was queried to determine whether the patient had volunteered to participate. Inclusion criteria for the EHI group were as follows: patients volunteered to participate before onset of the heat illness episode, heat illness occurred between July and September 2000 in association with a training run similar to that performed by the E group, and patients presented at the BMC with 

\[ T_{es} \approx 39.2^\circ C \].

The EHI group consisted of 10 men and 1 woman. Heparinized 3-ml blood samples were obtained immediately after 

\[ T_{es} \]

measurement at the BMC (time 0; ~20 min after EHI onset), after the tepid shower, and ~2 and 24 h later.

At each sampling point for E or EHI, 100-µl aliquots of whole blood were added to one of two mixtures containing fluorescently labeled antibodies (Coulter), each previously titrated to obtain optimum fluorescent signaling with lowest antibody volume. One mixture consisted of 10 µl each of antibodies to CD5, CD8, and CD69 and 5 µl of antibody to CD4; the other mixture contained 10 µl each of antibodies to CD5, CD56, and CD69 and 20 µl of antibody to CD19. All antibody-treated samples were vortex mixed and incubated for 15 min at room temperature in the dark. Red blood cells were lysed, and white blood cells (WBC) were fixed by addition of 500 µl of Optilyse (Coulter). After the samples were vortex mixed and incubated at room temperature for 10 min in the dark, they were diluted with phosphate-buffered saline (500 µl). Samples were then shipped on ice by overnight express carrier to the US Army Research Institute of Environmental Medicine and held at 4°C until analyzed by flow cytometry. Analysis was performed within 4 days of sample collection on an Epics XL flow cytometer using Tetra-one software for four-color analysis (Coulter). Primary gating of lymphocytes was based on intensity of CD45 fluorescence in conjunction with cell size and granularity as reflected by light forward and side scatter, respectively. Cells within this gate were then discriminated by CD3 fluorescence (positive or negative) in conjunction with other specific cell surface markers to distinguish the various lymphocyte subsets. T helper (CD4), T suppressor (CD8), T natural killer (CD56), and B cell (CD19) lymphocyte subsets were identified, and the CD4-to-CD8 ratio was calculated. Because PHA stimulation reduces CD3 expression (18), primary gating for PHA-exposed samples was based on the CD5-positive lymphocyte population. From the CD5 population, the percentage of each lymphocyte subset expressing the CD69 cell surface marker was determined. In addition, fluorescent intensity of CD69 cell surface markers on CD4 and CD8 (EHI = 5 men and 1 woman; E = 3 men and 3 women) lymphocytes, as well as the CD56 (EHI = 3 men; E = 3 men) lymphocytes, was determined. Fluorescence was expressed as the geometric mean of the fluorescent intensity.

At each sampling period, blood samples were also obtained for clinical chemistry and hematology measurements. These samples were part of the standard clinical analysis for suspected EHI patients at the BMC. A Beckman Coulter MD2 analyzer was employed for complete blood count and measurements of red blood cells, hematocrit, and hemoglobin, and a Beckman Coulter CX-7D analyzer was employed for the various tissue enzymes and other clinical chemistries. In addition, height, weight, and scores on physical fitness tests for all subjects were obtained from US Marine Corps personnel and training records.

Values are means ± SE. Comparisons between E and EHI at time 0, 2 h, and 24 h employed a two-way analysis of variance with repeated measures followed by Tukey’s post hoc analysis. A general linear model adjusted for the unbalanced design relative to the differences in subject numbers between EHI and E. Pre-E and E time 0 comparisons used paired t-tests; pre-E, E time 0, and EHI time 0 comparisons employed analysis of variance followed by Tukey’s post hoc analysis. Significance was set at P < 0.05.

RESULTS

Table 1 shows subject characteristics for the E and EHI groups. The E and EHI populations were young. Significant differences in body mass index (BMI) were noted. However, these differences did not significantly alter times for the 1.5-mile run, a performance measure for all entering recruits. Number of training days was significantly greater at EHI onset.
A comparison of the clinical indexes for E and EHI is found in Table 2. EHI mean body temperature was 40.4 ± 0.3°C, with a range of 39.2–41.7°C. This temperature was significantly greater than that for the E group (38.6 ± 0.2°C) after completion of the running exercise. One patient was delirious in the BMC; however, the only mental-status impairment recorded in any other patient was slow mentation. Thus most were diagnosed with EHI. WBC count at EHI time 0 was significantly elevated compared with pre-E, but not E time 0. No significant differences among pre-E, E time 0, and EHI time 0 were noted for WBC type. Although no significant differences in uric acid concentrations were noted, creatinine was significantly elevated at EHI time 0 compared with pre-E or E time 0. Although lactate dehydrogenase and aspartate aminotransferase were significantly elevated at EHI time 0, other enzyme markers of tissue injury, although elevated, were similar to pre-E or E time 0.

Preliminary study demonstrated no significant differences in lymphocyte subset distribution or CD69 response among processed blood samples (i.e., fixed and antibody exposed) held at 4°C for 1–5 days (data not shown). Thus delays (majority of samples analyzed within 2 days and none no later than 4 days after processing) in flow cytometric analysis due to shipment from Parris Island to the US Army Research Institute of Environmental Medicine did not significantly contribute to the findings.

Absolute count for CD3+ lymphocytes from which lymphocyte subset discrimination was conducted was significantly higher at EHI time 0 than pre-E or E time 0. However, compared with pre-E or E time 0, this elevation in CD3+ count represented a significantly reduced percentage of the lymphocyte gate as defined by cell CD45 fluorescent intensity, size, and granularity (Fig. 1A). There was no significant difference in the CD4 count between EHI and E, but percent CD4 for EHI was significantly reduced compared with pre-E or E time 0. Moreover, percent CD4 at E time 0 was significantly elevated compared with pre-E (Fig. 1B).

At no sampling point were there significant differences in percent CD8 for comparisons between EHI and E (data not shown). However, CD8 count was significantly greater at E time 0 for EHI than for E (Fig. 1C). In EHI, CD56 was significantly elevated in count and percentage at time 0 compared with E time 0 or pre-E (Fig. 1D). The CD4-to-CD8 ratio was significantly reduced in EHI compared with pre-E or E time 0 (Fig. 1E). At all sampling points, B lymphocytes (CD19) showed no significant differences in count or percentage between EHI and E (data not shown). When significant differences between EHI and E were noted at time 0 for percentage, count, or CD4-to-CD8 ratio, these distinctions rapidly disappeared, such that, by 24 h, values were quite similar to pre-E values (Fig. 1, A and C–E; data not shown for percentage).

Background values (0 µg/ml PHA) for percent CD69 expression by CD4 lymphocytes were low (Fig. 2A). For CD8 lymphocytes, background CD69 was significantly higher for EHI time 0 than pre-E (Fig. 2B). Exercise also influenced CD8 background CD69 expression, as revealed by the significant elevation between pre-E and E time 0. After exposure to 10 µg/ml PHA, pre-E CD4 and CD8 percent CD69 expression were greatly elevated, but after exercise (E time 0) the capacity of CD4 lymphocytes to respond to PHA was significantly reduced. Responses of CD4 and CD8 lymphocytes from EHI patients to the same PHA concentration were significantly suppressed compared with pre-E and all post-E sample times. With a stronger PHA stimulus (20 µg/ml), EHI CD4 and CD8 lymphocytes showed enhanced percent CD69 expression, but this enhancement was still significantly less than that shown by E controls at all sample times. As with 10 µg/ml stimulation with 20 µg/ml PHA revealed a significant reduction in percent CD69 expression for CD4 at E time 0 compared with pre-E. The responses of CD56 (Fig. 2C) lymphocytes to PHA stimulation were qualitatively similar to those of CD4 and CD8 lymphocytes, except E and EHI lymphocytes were not significantly different.
at time 0, and the response of this lymphocyte subset to stimulation with 20 μg/ml PHA did not differ between E and EHI. In addition, EHI time 0 CD56 lymphocytes had high background CD69 levels that were significantly elevated compared with pre-E.

In the absence of PHA (0 μg), no significant differences in CD69 fluorescent intensity between E and EHI for CD4 and CD8 lymphocyte subsets were noted (Fig. 3, A and B). With PHA stimulation (10 or 20 μg), CD4 and CD8 for EHI showed significantly reduced CD69 fluorescent intensity at various times, with CD8 demonstrating a more consistent reduction in fluorescent intensity. CD56 for EHI demonstrated a significantly elevated CD69 fluorescent intensity at 2 h compared with E in the absence of PHA (Fig. 3C). However, in the presence of 10 μg/ml PHA, CD69 fluorescent intensity for EHI CD56 lymphocytes at 2 h was significantly reduced compared with E. Although generally reduced, no significant differences were apparent in CD69 fluorescent intensity between EHI and E for CD56 lymphocytes exposed to 20 μg/ml PHA.

**DISCUSSION**

Previous heatstroke studies describe immune system disturbances (1–3, 12, 13). However, these studies compare sedentary controls with patients experiencing heatstroke while participating in the hajj, the annual pilgrimage to Mecca. For the past two decades, it has been recognized that heatstroke occurs in two forms, classical and exertional, depending on whether the illness is associated with prolonged or strenuous exer-
Fig. 2. Comparison between patients with EHI and subjects performing similar exercise (E) for phytohemagglutinin (PHA)-induced percent CD69 expression by CD4 helper (A), CD8 suppressor (B), and CD56 natural killer (C) lymphocytes.
Fig. 3. Comparison between patients with EHI and subjects performing similar exercise for PHA-induced CD69 mean fluorescent intensity by CD4 helper (A), CD8 suppressor (B), and CD56 natural killer (C) lymphocytes.
Exercise. Heatstroke during the hajj is often described as being of the classical type, but the associated rituals (16) require sufficient physical exertion that heatstroke during the hajj is of a mixed type, with features of the classical and the exertional forms (2). Because exercise alone influences immune system parameters (7, 8, 14, 21–27, 29–31, 35, 36), exercise contributions to the findings in heatstroke patients during the hajj are unclear. Moreover, the ages of these heatstroke patients (mean 43.2–58.1 yr reported in various studies) suggest that chronic premorbid conditions may have confounded the findings. In the present study, although differences in gender ratio and number of training days between E and EHI (Table 2) represented limitations, the youth (<20 yr) of the participants and their ability to meet the physical standards for acceptance as recruits likely excluded underlying chronic disease from influencing the findings. Most importantly, only EHI cases occurring during military training that was similar in type (running) and length (1.75–2 miles) to that performed by controls were included in the comparisons. Moreover, because exercise alone or heatstroke similarly influences immune cell dynamics, this study of EHI provided an opportunity to determine whether heat stress of a severity between that of exercise and heatstroke also influenced immune system parameters when controlled for the effects of exercise.

Although differences in gender ratio likely contributed to significantly larger BMI for EHI than for E (Table 1), high BMI as a risk factor for EHI (9) may also account for such group differences. The significant elevation in some tissue enzymes for EHI vs. E (Table 2) suggested a greater degree of tissue injury in the EHI group. Although EHI patients had more days of military training than E (Table 1), this likely did not account for differences in tissue enzymes, because serum enzyme levels at the end of a run decrease over the course of training (unpublished data). Muscle damage was suggested by the elevation in creatinine in the EHI cases (Table 2). However, because not all E HI urine acid values fell within the normal range, decreased urinary function cannot be excluded as contributing to this finding.

Adjustment of WBC and lymphocyte subset counts or tissue enzyme levels for changes in blood or plasma volume, as determined from hematocrit and hemoglobin, was not employed. Because E blood collections were obtained under field conditions during training and EHI blood collections were obtained from patients at the BMC, the variables of tourniquet application, arm position, and body posture make determination of plasma or blood volume unreliable using hematocrit and hemoglobin (28). However, as shown in Table 2, plasma sodium for pre-E, E time 0, and EHI time 0 was not different, and no differences were noted 24 h after E or EHI (data not shown). This, in conjunction with military doctrine of regulated breaks in which trainee water consumption is observed by the instructor, suggests that the influences of dehydration were not significant.

In the absence of heat illness, the running exercise itself was shown to influence immune system parameters. For example, WBC count for EHI time 0 was significantly increased compared with pre-E but not compared with E time 0 (Table 2). Pre-E and E time 0 comparisons demonstrated significant differences in percent CD4 (Fig. 1B), percent CD56, and CD56 count (Fig. 1D). Although CD56 count was significantly decreased (Fig. 1D), CD4 (Fig. 1B) and CD8 (Fig. 1C) counts showed a trend toward reduced numbers at E time 0 compared with pre-E. This contrasted with the reported significantly increased numbers for these lymphocyte subsets after exercise (26, 27), which suggested a difference in the duration and/or intensity of exercise between the present study and previous studies. Comparisons between pre-E and E time 0 for PHA-stimulated CD69 expression revealed a significant decrease for CD4 (Fig. 2A) but not for CD8 (Fig. 2B) or CD56 (Fig. 2C). Because these lymphocyte subsets have been previously shown to have a significantly suppressed response to PHA after exercise (23, 36), the absence of significant exercise-induced suppression in CD69 expression by CD8 and CD56 lymphocytes further indicated that exercise in the present study was perhaps not as intense as that in earlier studies. Irrespective of possible differences in exercise intensity, because some changes in immune cell dynamics were recorded, comparisons with controls performing similar exercise were necessary to fully distinguish the influence of EHI pathogenesis on immune system alterations.

As illustrated in Table 3, EHI immune cell dynamics were generally, but not always, similar to those previously described for exercise alone (absence of heat illness) or heatstroke. For example, leukocytosis (Table 2), limited effects on CD19 lymphocytes (data not shown), and significant increases in CD8 (Fig. 1C) and CD56 (Fig. 1D) lymphocytes resulted with EHI. This is also noted in exercise (26, 27, 36) or heatstroke (2, 13). Similar to exercise (7, 22, 26, 27, 36), EHI was associated with increased numbers of CD3 lymphocytes (Fig. 1A); however, this lymphocyte subset is unchanged in heatstroke (13). CD4 lymphocyte numbers are increased by exercise (26, 27, 36) and were unchanged after EHI (Fig. 1B), whereas they are decreased in...
heatstroke (2, 13). The CD3 and CD4 lymphocyte relation among these three exertional conditions that range from absence to presence of various severities of heat illness tended to illustrate the middle-ground position for EHI, because the CD3 or CD4 dynamic for EHI was more similar to that reported for the exercise or heatstroke condition, respectively (Table 3).

Similarity in immune cell dynamics among exercise, EHI, and heatstroke likely reflect a common denominator of hyperthermia in association with some level of physical exertion. Dissimilarity may reflect one or more of the following factors: differences in the age and premorbid health of the EHI patient or exercise subject populations, differences in exertion level of controls in various studies, differences in the degree of hyperthermia, and differences in sampling time at the end of exercise compared with the elapsed time before medical attention in the case of EHI. In studies of exercise (7, 8, 22, 24, 26, 27, 29, 36), subjects are young and sampling occurred immediately at the end of exercise, with comparisons made to controls at rest or performing similar exercise under a thermal-clamp design. In the present EHI study, patients were young, largely diagnosed as exertional heat exhaustion or injury, admitted to the BMC 10–15 min after the onset of their illness, and compared with controls performing similar exercise. By contrast, studies of heat illness patients from the hajj (2, 4, 5, 12, 13) were at or near middle-age, reported as heatstroke, admitted to the hospital in an average time <2 h after onset (4), and compared with sedentary controls.

Lymphocyte or lymphocyte subset alterations in this EHI study were of short duration. By 2 h, EHI alterations were hardly apparent compared with E, and by 24 h, values for E and EHI were equivalent to pre-E values (Fig. 1, A–D). Because EHI 24-h values return to the pre-E levels, the significant alterations noted in EHI compared with E at time 0 did not likely represent conditions before EHI pathogenesis.

Significant elevation in percent CD8 (Fig. 2B) and CD56 (Fig. 2C) background (0 µg PHA) CD69 expression at EHI time 0 vs. pre-E indicated a mitogen response during development of EHI. However, the possibility of this condition preexisting in the EHI population (e.g., recent infection or tissue injury) cannot be excluded. Irrespective of higher background levels, these lymphocyte subsets still experienced a significantly reduced PHA response in EHI compared with E (Fig. 2, B and C). CD4 lymphocytes (Fig. 2A) from EHI patients also showed a significantly reduced percentage of cells expressing CD69 compared with E. Mean CD69 fluorescent intensity illustrated the EHI influence on the relative density of CD69 cell surface markers per cell noted in the presence or absence of PHA. CD4 and CD8 in EHI demonstrated a reduction in CD69 density after stimulation with PHA (Fig. 3, A and B). Similar robust findings were not as apparent for CD56 (Fig. 3C); however, this may reflect the reduced number (n = 3) of EHI patients tested for this particular lymphocyte subset. Reductions in CD69 density per cell and percentage of lymphocyte subsets expressing CD69 suggested a suppressed T-cell mitogen response. Suppressed T-cell mitogen responses also occur after exercise without heat illness (27, 36). Although T cell mitogen response suppression has not been determined for heatstroke, Bouchama et al. (2) concluded that immune cell distribution disturbances might contribute to the increased susceptibility to infection after heatstroke (9, 17, 19, 34). However, this enhanced susceptibility perhaps related to immune cell distribution disturbances, as well as mitogen response suppression, may be a trade-off to reduce further acute-phase injury induced by adverse immune activity.

The present study demonstrated that immune system disturbances with EHI were distinguishable from the contributions of exercise. Because exercise conducted under a thermal-clamp design to reduce core temperature elevation substantially blunts many immune system alterations (7, 26), the significantly higher body temperature in EHI than in E (Table 2) may explain, in part, why immune cell dynamics in EHI were distinguishable from those in E. As recently reviewed by Shephard (30), increases in exercise-related hyperthermia generally move in parallel with elevations in the stress hormones that influence immune cell dynamics. This relation between hyperthermia and factors influencing immune cell dynamics likely contributed to the leukocyte changes noted in EHI and perhaps accounts for the similarity of the findings, not only to heatstroke (2, 13) but also to exercise in the absence of heat illness (23, 26, 27, 36). This suggests that heat stress of varying severity may share a similar injurious process in regard to immune system disturbances.

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DISCLOSURES

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