Increased serum nIgM in voluntarily physically active rats: a potential role for B-1 cells

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HABITUAL, MODERATE PHYSICAL ACTIVITY is associated with a lower risk of cardiovascular disease, diabetes, cancer, and infectious illness (1, 17, 29). One way physical activity may impact health is through changes in the immune system. There is evidence that habitual physical activity modulates many components of immunity. For example, NK cell cytotoxicity in rats, mice, and humans (10, 21, 28, 29, 31), T cell proliferation, and interleukin (IL)-2 production in mice (33), and macrophage function in mice (24, 33, 36) are all potentiated with exercise. Therefore, exercise can modulate IgA levels. The majority of this work has involved measuring changes in salivary IgA (sIgA) in the absence of a laboratory antigenic challenge. Therefore, constitutive, as well as potential environmental, antigen-driven responses are studied. Moderate, habitual exercise can increase sIgA, whereas intense exercise can decrease it (17). Interestingly, B-1 cells (19, 22), in addition to B-2 cells, produce mucosal IgA. Thus examination of mucosal IgA levels in humans examines the contributions of both cell types and indicates that perhaps the cells being modulated with exercise are B-1 cells, since there is little evidence of antigen-driven Ig levels being modified through exercise alone.

Although these data are intriguing, human studies using sIgA as an immunity status marker can have
limitations. For example, because there can be as much as 15-fold differences in salivary rates between individuals (6), salivary flow rate has to be accounted for (17). In addition, there appears to be a difference in secretion due to age and perhaps even to the season. Also, sIgA production can be stress sensitive and, therefore, could be affected by participant life stresses outside of laboratory control (25). Furthermore, it is challenging to compare humans with one another, as every person has a large and variable antigenic exposure from day to day and throughout life.

Many of these confounding factors inherent to investigation of human nonantigen-drive Ig production can be removed by examining serum concentrations of nIgM levels in a pathogen-free animals in a barrier facility (8, 13). Circulating levels of nIgM are a good measure of B-1 cell function in pathogen-free animals because nIgM comprises ~90% of the circulating IgM (5). Although nIgM does contribute to circulating IgM in humans, it is not possible to easily detect the unique contribution of B-1 cell nIgM due to the large and variable antigen exposure history of humans, and thus the variable contribution of B-2 cell antigen-driven IgM to circulating IgM.

The role of B-1 cells in immune function has received renewed interest in immunology (4). B-1 cells are phylogenetically primitive B cells that primarily reside in the peritoneal cavity, are self-replenishing, and produce polyclonal nIgM in the absence of antigen. B-1 cells produce the majority of nIgM. nIgM is a vital first line of defense against bacteria and viruses and can bind tumor antigens and facilitate tumor killing (9). During a challenge, B-1 cells can migrate to other areas of the body, such as the spleen, to aid in host defense. Also, nIgM is critical to the initiation of the classical complement pathway (8, 13, 18). There is evidence that nIgM is also important in aiding B-2 class switching to make high-affinity antigen-specific IgG (5). No previous research has specifically explored the effect of physical activity on circulating nIgM. Thus examination of constitutive B-1 cell nIgM may prove to be an important, and thus far unexplored, area of exercise influence on immunity.

The purpose of this investigation, therefore, was to test the impact of activity status on nonantigen-driven Ig vs. antigen driven-Ig by using pathogen-free, adult Sprague-Dawley rats. Pathogen-free rats were housed in a barrier facility with either a locked (home-cage control) or mobile running wheel (physically active). Animals with the mobile wheels maintained a physically active lifestyle by running voluntarily every day from 1 to 8 wk. The impact of physical activity on antigen-driven Ig responses was tested in rats that were immunized with the benign protein KLH. The following studies tested the hypothesis that voluntary freewheel running would selectively elevate B-1-derived nIgM but not B-2-derived anti-KLH Ig. B-1 cells constitutively release nIgM, and a large number of these cells reside in the peritoneal cavity. Thus the effect of freewheel running on peritoneal fluid concentrations of nIgM was also examined. Additionally, to further test whether any effects found were due to running, per se, rather than to a general effect of an enriched environment, both B-1 (nIgM) and B-2 (anti-KLH Ig) levels from rats in living in an enriched vs. standard caging environment were also tested.

METHODS

Animals. Male Sprague-Dawley pathogen-free rats (Harlan, 290–315 g), 3–4 mo of age, were used (10 rats/group). Animals were housed singly in Plexiglas cages with attached running wheels in a biofiltered room (BioBubble, M501A) to ensure pathogen-free conditions. Subjects were maintained on a 12:12-h light-dark cycle (lights on 0700–1900). Animals remained undisturbed for 1 wk before the onset of the studies. The care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

General procedures. The general experimental design can be found in Fig. 1. Rats were either allowed to run (physically active) or remained in their home cage with locked wheels (sedentary). To test the impact of long-term running on nIgM, total IgG, and serum protein, blood samples were taken from the tail vein on days 0, 5, 7, 14, 28, and 35 after running onset. To test the long-term impact of running on antigen-driven Ig, animals were allowed to run undisturbed for 28 days, immunized with KLH, and allowed to continue running until day 56. Blood samples were taken from the tail vein on days 35, 42, 49, and 56 after running onset. Anti-KLH IgM, anti-KLH IgG, and total serum protein were measured. After 6 wk of running, animals underwent peritoneal lavage.

Fig. 1. The following experiments were conducted to test the hypothesis that physical activity selectively affects B-1 but not B-2 cells. Rats were tested after either long-term (42–56 days) or short-term running (7 day) regimens or housed in an enriched environment (56 days). Adult male Sprague-Dawley rats (10 rats/group) were housed with either a mobile running wheel (physically active) or with a locked running wheel (sedentary) or with rat toys (enriched). Blood samples (B) were collected on various days after running onset, housing enrichment, and/or keyhole limpet hemocyanin (KLH) immunization.
to assess IgM levels. To test how quickly wheel running impacts Ig, animals received either no antigen or were immunized with KLH on day 0 of running onset. Physically active rats were allowed to run for 7 days. Blood samples were taken from the tail vein on days 3, 5, and 7 after running onset. Either nIgM, total IgG, and serum protein (nonimmunized) or anti-KLH IgM, anti-KLH IgG, and total serum protein (KLH immunized) were measured.

Running activity. Rats were individually caged in Nalgene Plexiglas cages (45 cm × 25.2 cm × 14.697 cm) with a stainless steel, open running wheel attached (46.8 cm × 24.9 × 34.2 cm). Physically active rats had a mobile running wheel and chose to run every day throughout the duration of the study. Sedentary controls were housed in the same environment except that the running wheel was locked and remained immobile throughout the duration of the study. Voluntary freewheel running was the chosen modality because, in contrast to forced treadmill training, voluntary freewheel running does not produce negative adaptations that are indicative of chronic stress in rats (26, 27). The caging environment meets National Institutes of Health floor-space standards for a single rat (26, 27). Daily running distance (C) was monitored by computer with the VitalView Automated Data Acquisition System (Sunny River, OR). Rats were weighed weekly.

Enriched environment. One potential explanation for the effect of voluntary wheel running is that the presence of a wheel may simple create an enriched environment and that any effect found is due to enrichment rather than to running. To determine whether an enriched environment impacted serum Ig levels, rats (n = 12, 6/group) were housed individually in cages with toys. Each animal had one plastic flowerpot 4 in. in diameter and 6 in. in height and two toilet paper rolls. The flowerpot and toilet paper rolls were autoclaved before introduction into the rats’ home cages and were replaced daily as needed. This type of enriched environment has been previously reported to increase cell proliferation in the brain (34).

KLH immunization. Rats were given an intraperitoneal injection of 200 μg of soluble KLH (in 50% glycerol) in saline (Sigma Chemical, St. Louis, MO). KLH was chosen because we have previously reported that this antigen triggers a robust B-2 antigen-specific Ig response in the absence of any sickness (14, 27).

Tail vein blood sampling. Blood samples, taken via the tail vein, were obtained within 2 min of touching the cage. Animals were wrapped in a cloth and then gently restrained by a Velcro strap anchored to a table. The tail was exposed, and a small nick with a no. 15 scalpel blade was made. Blood (~150 μl) was quickly milked from the tail vein. Animals were then returned to their home cages. Samples were allowed to clot at room temperature for 30–45 min and were then spun at 4,000 revolutions/min for 30 s. Serum was removed, and samples were frozen at −20°C until assays were performed.

Peritoneal lavage. Animals were gently removed from their cages and, within 1 min after removal, were decapitated by using a guillotine. Heat-sterilized tools and sterile pipettes were used. The abdominal region was sprayed down with ethanol (70%, Sigma Chemical), and the skin was removed. A small hole was cut in the abdominal wall, and 2 ml of cold sterile (balanced salt solution [Sigma Chemical] was added. The abdominal region was then massaged for 15 s. The fluid was retrieved via a pipette, placed in a sterile snap cap, and put on ice. The fluid was spun at 3,000 revolutions/min for 30 s, and the supernatant was removed for assessing IgM.

Enzyme-linked immunoabsorbent assays: nIgM, total IgG, anti-KLH IgM, and anti-KLH IgG. nIgM and IgG, and anti-KLH IgM and anti-KLH IgG enzyme-linked immunoabsorbent assays (ELISAs) were performed by using 96-well plates (Dunque). For nIgM or IgG ELISAs, plates were coated with goat anti-rat IgM or IgG (Cappel) and incubated overnight at 4°C. Plates were then washed twice with phosphate-buffered saline and 0.05% Tween 20 (PBS-T; Sigma Chemical) and blocked with 5% bovine serum albumin (Sigma Chemical) overnight at 4°C. Plates were washed three times in PBS-T.

Rat IgM and IgG (Pharmingen) were used to generate a standard curve. The standard ranged from 10.4 to 333.3 μg/ml. Samples assayed in duplicate were diluted (1:4,800 and 1:9,600 in PBS-T) at concentrations that placed them on the linear portion of the standard curve. Plates were covered with plate sealers and incubated at room temperature for 4 h. Plates then were washed three times with the plate washer with PBS-T. Goat anti-rat IgM or IgG with alkaline phosphate tag was then added (Cappel), and plates were incubated another hour. Plates were washed three times with PBS-T, and p-nitrophenyl phosphate liquid substrate was added (Sigma Chemical). Plates incubated in the dark until the most concentrated standard read between 1.3 and 1.5 optical density on the plate reader (Molecular Devices) at 405 nm. For the peritoneal samples, the same standard curve method was used. Samples were diluted 1:32 and 1:64 in duplicate.

Anti-KLH ELISA plates were coated with KLH at 0.5 μg/ml and incubated were overnight at 4°C. Plates were washed twice with PBS-T and blocked overnight with 5% bovine serum albumin. Subsequent steps used the same reagents as IgM and IgG ELISAs. Incubation periods were the same but were in a warm oven at 37°C. Positive and negative control sera were run on every plate. The positive control is pooled sera from KLH-immunized rats. This sample is then used to control for any plate-to-plate variability. Data are presented as a proportion of sample optical density to plate positive optical density. Negative control sera are comprised of pooled samples from nonimmunized controls. These are run to control for nonspecific binding. The negative control samples in the anti-KLH ELISAs run from 0.05 to 0.2.

Total protein. Total serum protein was determined from serum via the Bradford assay, as described previously (14). Briefly, 10 μl of 1:200 dilution of sample were pipetted into a 96-well plate (Dunque), and 250 μl of Bradford reagent were added. Plates were incubated at room temperature for 10 min and then read at 590 nm on the plate reader.

Statistical analysis. StatView was the statistical package used in all analyses. Repeated-measures ANOVA was used on weight, Bradford, total IgG, total IgM, and anti-KLH IgM. Regression analysis was done by simple regression on total running distance to total protein or total IgM. Post hoc analysis by Fisher’s protected least significant difference test was done on total IgM. Significance for all tests was set at P = 0.05.

RESULTS

Weight. Long-term voluntary freewheel running has been previously reported to modestly reduce body weight gain (~5%) in male Sprague-Dawley rats (27). In the present studies, rats that ran for 42 days and were not immunized with KLH did not have differences in body weight gain (P > 0.05); both groups gain weight over time (P < 0.001). There was a trend for significant time-by-activity interaction (P = 0.11), sug-
gesting that physically active rats were gaining slightly less body weight across time. Rats that ran for 56 days and were immunized with KLH did have a statistically significant reduction in body weight gain across time ($P < 0.05$). The reduction in body weight gain was once again modest, ~5%. Short-term physical activity (7 days) did not effect daily body weight gain in rats that were not immunized with KLH ($P > 0.05$) or in rats immunized with KLH ($P > 0.05$).

Running distances. The average weekly running distances are presented in Table 1. As has been previously reported (27), male Sprague-Dawley rats increase their running distances across time. In the present studies, the average weekly running distances in the first week were 5.1–5.5 km/wk. By the end of the studies, both groups of long-term running rats had more than doubled their weekly running distances (16.7–19.6 km/wk). Short-term runners ran 2.3–3.3 km/wk. To investigate whether running distance influenced serum protein or nIgM production, simple regression analyses were preformed. Pearson correlational analyses were done between total distances run and total serum protein, and distances run and total nIgM. As shown in Table 2, there were no reliable correlations between the amount of running and the amount of serum protein or nIgM.

**Table 1. Average 7-day running distance over 42–56 days**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
<th>Day 49</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>No KLH</td>
<td>5.5 ± 1.5</td>
<td>7.3 ± 2.7</td>
<td>12.6 ± 5.4</td>
<td>13.3 ± 9.3</td>
<td>18.8 ± 10</td>
<td>19.6 ± 12</td>
<td>Study end</td>
<td>Study end</td>
</tr>
<tr>
<td>KLH</td>
<td>5.1 ± 2.1</td>
<td>8.2 ± 4.7</td>
<td>15.4 ± 10</td>
<td>11.6 ± 8</td>
<td>11.3 ± 4.2</td>
<td>14.9 ± 6.4</td>
<td>16.5 ± 10</td>
<td>16.7 ± 9.5</td>
</tr>
</tbody>
</table>

Values, in km, are means ± SE. Adult, male, Sprague-Dawley rats (10 rats/group) were housed with either a mobile running (physically active) or a locked running wheel (sedentary) for 42 of 56 days. Rats were either immunized with keyhole limpet hemocyanin (KLH) or did not receive any.

**Table 2. Simple regression comparing km run with total circulating protein and km run with nIgM in non-KLH immunized rats**

<table>
<thead>
<tr>
<th>Length of Run, days</th>
<th>Protein</th>
<th>nIgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>0.24</td>
<td>0.56</td>
</tr>
<tr>
<td>7</td>
<td>0.23</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Simple regression analyses of total kilometer (km) run to total protein concentration and total km run to total serum protein concentration and total km run to total serum nIgM concentrations were performed in nonimmunized rats that ran for either 42 or 7 days. No statistically significant correlations (R) were found, suggesting that, in these experiments, running distances did not predict natural IgM (nIgM) elevations.

Short-term running effect on nIgM, IgG, and protein in nonimmunized rats. As shown in Fig. 5A, freewheel running elevated serum concentrations of nIgM levels [$F(1,12) = 4.5, P = 0.05$]. Post hoc analysis showed significantly higher elevations in runners on day 7 ($P = 0.02$). Short-term freewheel running had no effect on total IgG (Fig. 5B) and serum protein (data not shown).

Short-term running effect on anti-KLH IgM, anti-KLH IgG, and protein in KLH-immunized rats. As shown in Fig. 6, freewheel running had no effect on serum concentration of anti-KLH IgM (Fig. 4A), anti-KLH IgG (Fig. 4B), and protein (Fig. 4C). KLH immunization elevated antigen-specific Ig. The pattern of Ig response is similar to that previously reported (14, 27), with anti-KLH IgM peaking after 7–10 days, whereas anti-KLH IgG climbed for several weeks after KLH immunization.

**DISCUSSION**

This is the first study examining B-1 nIgM production as measured by sera IgM in pathogen-free, free-wheel-running rats. These experiments demonstrate that physical activity in rats selectively elevates nIgM...
(B-1) and not antigen-specific Ig (B-2). The physical activity induced elevation of nIgM, which is detectable as early as 7 days and persists for up to 42 days after running onset.

The effect of physical activity was specific to circulating nIgM. Just as antigen-driven Ig was not affected, neither was circulating IgG nor total serum protein. This suggests that the effect of physical activity on nIgM was not due to hydration status or hemodynamic changes. Additionally, an enriched environment did not alter rat serum IgM, IgG, or anti-KLH IgM. The lack of effect of environmental enrichment suggests that it is physical activity, not the addition of the wheel, per se, that caused these changes in nIgM. Additionally, after 42 days of running, physically active animals have more IgM in the primary residence compartment of B-1 cells, the peritoneal cavity.

As B-1 cells primarily make only IgM (5, 18, 30, 35), no changes in circulating IgG between groups is consistent with the hypothesis that B-1, not B-2, cells are impacted by running. Interestingly, there was not a correlation between distance run and sera albumin or a correlation between distance run and IgM (Table 2). This indicates that a minimum threshold of running is required to increase IgM and that the running distances and IgM are not linearly related. More work is required to determine what the minimum threshold of running is to obtain this effect.

Although the minimum threshold of running required to increase nIgM remains unknown, the present study does suggest that the effect is due to changes in physical activity and not to environmental enrichment. This can be concluded for several reasons. First, both the sedentary and the physically active animals had nearly identical environments. Both were housed with a running wheel; only the mobility of the wheel was different (locked vs. free). Second, housing animals in an environment that has been previously reported to

![Fig. 2. Adult male Sprague-Dawley rats (10 rats/group) were housed with either a mobile running wheel (physically active) or a locked running wheel (sedentary) for 42 days. Blood was collected via the tail vein on days 0, 5, 7, 14, 28, 35, and 42 after running onset. Serum levels of natural IgM (nIgM; A), IgG (B), and protein (C) were measured by using enzyme-linked immunosorbent assay (ELISA) and Bradford assays. Physical activity reliably elevated nIgM on days 14, 28, 35, and 42. *Significant difference, $P < 0.05$.](https://www.jap.org)
increase neuronal proliferation (flower pots and toilet paper tubes; Ref. 34) also did not affect antibody levels. Thus the state of physical activity seems to play a critical role in this effect.

What remains unclear, and cannot be determined with the present experimental approach, is which of the tested environments (sedentary vs. active) results in the “normal” physiological state. It would be easy to argue that the sedentary condition is the abnormal physiological state and that the physically active condition is the normal physiological state. Given this perspective, our results would be equally demonstrative of the potentially immunologically detrimental impact of a sedentary lifestyle. It is feasible to hypothesize that the lack of physical activity results in a decreased or suboptimal nIgM concentration.

B-1 cell production of nIgM has received renewed interest in immunology (4). nIgM plays an important role in many aspects of immune function and can be considered innate humoral immunity: innate, because it is in the body before pathogenic exposure, and humoral because it is an antibody. Increased nIgM could have several potential positive consequences for host defense. In humans, decreased nIgM is thought to play a role in the development of human neuroblastoma (11). nIgM helps coordinate many aspects of the immune response during bacterial and viral challenges. For example, mice without secreted nIgM die from a survivable bacterial infection (5) and, after viral infec-

Fig. 4. Adult male Sprague-Dawley rats (10 rats/group) were housed with either a mobile running wheel (physically active) or with a locked running wheel (sedentary) for 28 days, immunized with KLH, and allowed to continue running. Blood was collected via the tail vein on days 35, 42, 49, and 56 after running onset. Serum levels of anti-KLH IgM (A), anti-KLH IgG (B), and protein (C) were measured by using ELISA and Bradford assays. Anti-KLH IgM decreased across time, and anti-KLH IgG increased across time. Physical activity had no effect on anti-KLH IgM, anti-KLH IgG, or total serum protein. Means ± SE are shown.

Fig. 5. Adult male Sprague-Dawley rats (10 rats/group) were housed with either a mobile running wheel (physically active) or a locked running wheel (sedentary) for 7 days. Blood was collected via the tail vein on days 3, 5, and 7 after running onset. Serum levels of nIgM (A) and IgG (B) were measured by using ELISA. Physical activity reliably elevated nIgM 7 days after running onset. Values are means ± SE. *Significant difference, P < 0.05.
tion, have higher viral load than wild-type mice (13, 35). Thus higher nIgM levels conceivably could lead to faster bacterial and viral clearance rates. However, there may be a negative result as well, because excessive B-1 cell activity may increase the chance of developing an autoimmune disorder. Because nIgM tends to be slightly self-reactive, if there is an overabundance of nIgM, it might trigger an immune response to healthy tissues (3).

There are several potential factors that could be responsible for the effect of physical activity on nIgM production. For example, growth hormone (GH) and IL-10 are impacted by physical activity and are probably important for B-1 cell maintenance. GH is necessary for lymphocyte maintenance and proliferation. There are GH receptors expressed on B-2 cells, and GH is released by B-2 cells as an autocrine growth factor (7, 15). There is evidence that GH is increased postexercise in humans and rats (7, 15). Although it is unknown whether B-1 cells express and/or release GH, it is feasible to speculate that exercise-induced GH increase may affect B-1 cells, causing a nIgM increase. GH effects on B-1 cells is an area that needs further examination.

Similarly, more research examining the effect of physical activity on IL-10 production is needed. IL-10 is the major autocrine growth factor for B-1 cells (30), and after heavy exercise IL-10 rises (31). Perhaps after moderate physical activity there is a slight rise in IL-10 locally in the peritoneal cavity. IL-10 production in the peritoneal cavity of physically active animals would be an interesting aspect to explore.

Although numerous studies have demonstrated the effects of exercise on many components of the immune system, this is the first report to demonstrate a specific effect of physical activity on nIgM but not antigen-driven Ig in pathogen-free rats. The elevation in nIgM probably stems from increased activity of B-1 cells, because >90% of IgM measured in the serum of pathogen-free animals is from B-1 cells (5) and the concen-
tration of nIgM in the peritoneal cavity (a B-1 cell residence) is higher in physically active animals. B-1 cells and innate humoral immunity are important for host defense. The consequences of increased nIgM are most likely positive, because nIgM is a critical first line of defense against invading bacteria (5) and viruses (13). An increase in nIgM might result in faster pathogenic clearance. Furthermore, because B-1 cells also contribute significantly to mucosal immunity through IgA production (5, 12, 16, 23), it may be of interest to study B-1 cells, particularly in humans, where there are indications that a decrease in sIgA in athletes may increase susceptibility to upper respiratory tract infections (17). The potential health implications of serum nIgM changes are presently under investigation.

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REFERENCES


