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The Effect of Season on Inflammatory Response in Captive Baboons

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Abstract

Introduction—Highly seasonal animals demonstrate predictable changes in immune function that coincide with changes in photoperiod. Little is known about the effect of season on immune response in baboons. The objective of this study was to determine the effect of season on inflammatory response in baboons.

Materials and Methods—Peripheral blood mononuclear cell cytokine response following immune stimulation and serum markers of inflammation were assessed during each season in two groups of young male baboons; one housed under natural light; one in a controlled environment of 12 hours light:12 hours dark.

Results—A seasonal immune rhythm was evident in both groups, with a greater TNF- α and IL-6 response to stimulation and serum CRP concentration in June and September compared to December.

Conclusions—Season is an important experimental confounder and therefore time of year should be controlled when designing studies and analyzing data from immune studies in baboons.

Keywords

cytokine; hormones; photoperiod; circannual

Background

Adaptation to environmental conditions promotes survival advantage to animals in the wild. Many species of animals respond to annual changes in day length (photoperiod) to best coordinate physiological and environmental resources with anticipated energetic needs. For example, photoperiod cues are used by seasonal breeders to accurately time reproductive activity to ensure offspring arrive at a favorable time of year [4, 16, 39]. Changes in metabolism occur in concert with change in season in many species, with metabolic markers such as body weight, adipose energy stores, voluntary food intake, metabolic rate and heat production having annual rhythms [5, 6, 12, 15, 34]. Length and color of coat are synchronized with season [15, 19].

Seasonal changes in immune response have also been described in many species including rodents, birds, and fish [7, 8, 25–27, 29, 34, 40]. The prevalence of many infectious or

inflammatory diseases follows a seasonal pattern, although it is unclear if this is a cause or consequence of a variable seasonal immune response [10, 14, 18]. Down regulation of immune response during times of sparse nutrient availability may serve to divert metabolic resources towards processes necessary for maintenance of life. As a result, disease susceptibility would be greater in winter. Alternatively, immune function may be bolstered when animal to animal interactions and thus risk of infectious disease are greatest, such as during breeding season. Whatever evolutionary advantage is gained by seasonal regulation of immunity in the wild, in laboratory animals seasonal variation in immune responsiveness could have profound implications in herd health management as well as interpretation and design of experimental studies.

The baboon is considered an excellent model for pre-clinical testing of vaccines and immune response to pathogens [17, 23, 31, 33, 38]. Baboons and chimps are unique among nonhuman primates in that similar to people they have four classes of IgG and share a similar susceptibility to pathogens [2, 17, 32]. However little is known about the effect of season on immune reactivity in baboons or other non-human primates. Therefore the objective of this study was to determine the effect that season has on the response of peripheral mononuclear cells (PBMC) to immune stimulation. We *hypothesized* that baboons housed outdoors under natural environmental conditions would experience seasonal fluctuations in inflammatory response. Furthermore, we expected that the seasonal immune rhythmicity would be absent or blunted in animals maintained indoors under a constant ambient temperature and a schedule of 12 hours artificial light: 12 hours dark.

Materials and Methods

Animals

24 healthy, 2–4 year old male baboons were included in the study. All baboons were housed and cared for according to the standards detailed in the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996). Protocols for maintenance of the baboon colonies were approved by the University of Oklahoma Health Sciences Center (OUHSC) Institutional Animal Care and Use Committee. Group 1 (Natural Environment) consisted of 12 baboons that were group housed outdoors under natural light in large, 5300 square feet corrals of 60–80 animals. Group 2 (Controlled Environment) consisted of 12 baboons housed indoors under constant 12 hour light, 12 hour darkness in small groups with 3 animals/ cage. The indoor rooms were sealed so as to allow no light from entering the room during the dark cycle. The controlled environment was maintained at a constant temperature of 23 °C. After group assignment, animals were allowed 3 months to acclimate to environments after which blood was collected every 3 months; on Mar 15, Jun 15, Sept 15, Dec 15 $\pm$ 3 days. Animals in both groups were moved into a novel cage, immobilized by squeezing then sedated with intramuscular ketamine for blood collection. Hours of daylight (photoperiod) were recorded from the US Navy Oceanography website, http://aa.usno.navy.mil/data/docs/RS_One_Year.php for each sample collection date (Table 1). Mean temperatures for the month of collection were recorded for the nearest airport using data from the website, <http://www.wunderground.com/history/> (Table 1).

Immune Assays

Tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were measured using a non-human primate specific ELISA (U-Cytech, Utrecht) each with a lower limit of detection of 1 pg/ml. Samples with a concentration below detection were assigned a value of 0.5 pg/ml for statistical analysis. Serum C-reactive protein (CRP) was determined using a human specific ELISA (MP Biomedicals, Orangeburg NY), with a lower limit of detection of 0.1 mg/L.

Hormone assays

Serum cortisol was measured by radioimmunoassay (Coat-a-Count, Siemens Diagnostics, Deerfield, IL). ACTH and alpha melanocyte stimulating hormone (α -MSH) concentration was measured in EDTA plasma by radioimmunoassay (ALPCO, NH; MP Biomedicals, Irvine, CA, respectively). Human specific assays were validated for use in baboons by demonstrating linearity of diluted baboon serum samples and linearity of diluted pooled serum samples spiked with known concentrations of substrate.

PBMC stimulation

Whole blood was collected from baboons in heparin containing CPT vacutainer tubes (Becton, Dickinson and Company, NJ) for peripheral blood mononuclear cell isolation. PBMC were isolated within 3 hours of blood collection using the method recommended by the CPT tube supplier. Cells were washed twice in phosphate buffered saline (PBS) and plated at a density of 5×10^6 cells/ml in RPMI supplemented with 10% FBS, 2mM glutamine, 100 μ g/ml streptomycin, and 100U/ml penicillin (RPMI complete media) for treatment with immune stimulants, lipopolysaccharide (LPS) or CpG deoxyoligonucleotide. For LPS, cells were incubated at 37°C, 5% CO₂ in the presence of either 1 μ g/ml of LPS (O111:B4, Sigma, St. Louis MO) or an equal volume of PBS for 4 hours [21]. For CpG, cells were incubated at 37°C, 5% CO₂ in the presence of either 5 μ M of CpG (InvivoGen, San Diego, CA) or 5 μ M control oligonucleotide for 6 hours [21]. All stimulation assays were performed on the day of blood collection. Following incubation, media was collected and frozen at -80°C for later, batched cytokine analyses.

Statistics

Data were assessed for normality using the Shapiro-Wilk test and non-normal data was log10 transformed for analysis. Two-way repeated measures analysis of variance was used to determine the effect of the factors season and environment on log10 transformed data. When a significant difference in season was identified, Holm-Sidak multiple comparison procedures were used to determine the specific differences among seasons. Anti-inflammatory hormone concentration was compared to the magnitude of cytokine response by calculating a Pearson's coefficient of correlation using log10 transformed data. A *P*-value of < 0.05 was considered significant.

Results

C-reactive protein and IL-6 are well documented serum markers of inflammation in baboons and therefore chosen for study [21, 36, 37]. There was no difference in the serum CRP concentration between animals housed indoors in a controlled environment and those outside in a natural environment, therefore the effect of season was analyzed with the two housing groups combined. Serum CRP concentration varied by season ($P < 0.001$, Fig 1), with a significantly greater CRP concentration in March, June and September compared to December. Serum IL-6 concentration also varied by season ($P < 0.001$, Fig 1) as well as by housing ($P < 0.001$, Fig 1), with no interaction between the effect of season and housing environment. In the outdoor baboons there was a significantly greater IL-6 concentration observed in December compared to that in June ($P < 0.01$), while in the indoor animals IL-6 concentration was greater in September compared to March ($P < 0.001$) and June ($P < 0.01$). Although there was a weak but significant correlation between serum CRP and IL-6 concentration ($r = 0.26$, $P < 0.05$), the seasonal rhythm of CRP more closely approximated the observed rhythm of the PBMC cytokine response to stimulation (see below), while the IL-6 concentration in the outside animals was more similar to the ambient temperature with the highest concentrations occurring in December when the lowest mean temperature was

observed and the lowest concentration in June when the highest mean temperature occurred (Table 1).

Anti inflammatory hormones ACTH, α -MSH and cortisol were examined for seasonal rhythm and their correlation to inflammatory markers CRP and IL-6. ACTH and cortisol are the most well studied hormones that respond to stress with strong anti-inflammatory actions. Alpha- MSH is also a potent anti-inflammatory pituitary hormone. Both ACTH and α -MSH show circannual rhythm in seasonal animals and therefore represent potential regulators of seasonal inflammatory response [15]. Plasma ACTH concentration showed an effect of season ($P<0.01$, Fig 2), with a lower concentration in December compared to March or September. Both ACTH and cortisol concentration were lower in those animals maintained in a controlled environment ($P<0.001$, Fig 2). For cortisol the effect of environment was dependent on season ($P=0.04$), while there was no interaction between the two factors, housing and season, for ACTH (Fig 2). There was no effect of either season or housing conditions on plasma α -MSH concentration. As expected, plasma ACTH concentration was positively correlated to serum cortisol concentration ($r=0.6$, $P<0.001$) and α -MSH concentration ($r=0.25$, $P<0.05$), while there was no correlation between cortisol and α -MSH concentration ($r=-0.02$, $P=0.9$). Both serum cortisol concentration and plasma α -MSH concentration were positively correlated to serum IL-6 concentration ($r=0.28$, $P<0.01$) but not CRP. ACTH concentration was not correlated to either serum inflammatory marker.

The proinflammatory cytokine response (TNF- α and IL-6) of PBMC to inflammatory stimulants LPS and oligodeoxynucleotide CpG was affected by season ($P<0.001$, Fig 3). In both the indoor and outdoor baboons, the TNF- α response to LPS stimulation was greatest in September, whereas the IL-6 response was greatest in September in the indoor baboons and in June in the outdoor baboons although the difference between these two months in either the indoor or outdoor group was not significant. The cytokine response to stimulation by CpG was greatest in June for both the indoor and outdoor housed baboons, with a greater IL-6 response in June in the indoor baboons compared to those in a natural, outdoor environment ($P<0.001$). There was no correlation between anti-inflammatory hormone concentration and proinflammatory cytokine response.

Discussion

Immune function is a systemically integrated, complex physiological process. Investigations of immune function are frequently performed in simple organisms such as worms or flies [23] or lower vertebrates such as rodents and fish [8, 9, 13]. While convenient for isolating the influence of individual genes, this approach ignores the complexity of these processes as they exist in higher order animals. Therefore the translational value of data gathered in these simpler species is questionable. Baboons are uniquely well suited for the study of complex biological processes such as immune response due to their physiologic and genetic similarity to people [23, 32]. Identifying factors that predictably affect immune function in non-human primates is therefore critical to facilitate design of experimental studies and interpretation of experimental design.

In the present study we sought to examine the role of season on innate immune response by assessing cytokine release from PBMC and serum markers of inflammation in blood collected during different times of the year. Lipopolysaccharide, a ligand of toll-like receptor (TLR) 4 and CpG deoxynucleotide, a ligand for TLR9 were both included as immune stimulants of PBMC to assess whether activation of different receptors would have different seasonal patterns. Lipopolysaccharide is a component of the cell wall of gram negative bacteria, such as *E. coli* and *Salmonella*. Unmethylated CpG motifs occur in viral, bacterial, fungal and parasitic DNA, but not vertebrate DNA. Therefore, it is recognized as

foreign DNA and acts as a non-specific immune stimulant. Both c-reactive protein, an acute phase protein, and IL-6, a proinflammatory cytokine, were investigated as serum markers of inflammation [21, 36, 37].

We found that baboons housed under natural light and weather conditions had a strong seasonal pattern in cytokine release from PBMC following immune stimulation. Interestingly the pattern observed following CpG stimulation, with a peak response in June, differed from that observed following LPS stimulation which elicited the strongest response in September and June. Both stimulants evoked the weakest response during the winter months, December and March. In addition, serum CRP concentration was lowest during December, suggesting the decrease of PBMC cytokine release was reflective of a decreased *in vivo* inflammatory state. In contrast, serum IL-6 had a seasonal pattern opposite to what was observed in the IL-6 stimulation assay with the serum IL-6 concentration highest in December. Many cell types can contribute to the serum IL-6 pool including mononuclear cells. Therefore it is possible the high serum concentration is reflective of a high basal IL-6 production from peripheral blood mononuclear cells and further response to stimulation was limited by a baseline activity approaching maximum cytokine release.

Similar to what we observed in baboons, mononuclear cells from hamsters housed in short photoperiod (SD) conditions showed a decreased in α -TNF release following LPS stimulation compared to those from hamsters housed in long photoperiod (LD) conditions [7, 29]. Furthermore, mortality secondary to LPS exposure, was greater in hamsters exposed to LD, consistent with a greater cytokine response and subsequent septic shock [29]. Rats exposed to short days also showed a decreased TNF- α release in response to LPS stimulation when compared to those maintained in long days [30]. In contrast to what has been observed in rodents and baboons, in people α -TNF and IL-6 production in whole blood after LPS stimulation was lowest stimulation in samples collected in September compared to June, February or March [25]. Samples were not collected in December in this study. This study included 17 participants residing in an urban area of Greece with a mix of males and females as well as smokers and non-smokers. Therefore, the more heterogeneous study population, differences in the environmental conditions or the type of samples used in the stimulation assays may have all contributed to the disparity of results between the studies.

Although absolute day length is similar in March and September, in the northern hemisphere the March equinox marks the beginning of a lengthening of daylight hours, where as the autumnal equinox is the start of shorter days. Therefore our results suggest it is the change in the length of day not the absolute day length that correlates to seasonal change in immune function. Furthermore, a seasonal effect was observed in both the group housed outside and those housed inside, suggesting seasonal immune rhythm in the baboon is a self sustaining rhythm. Many circannual rhythms are free running, meaning environmental cues, such as photoperiod, aren't necessary to maintain the rhythm, only to synchronize the rhythm. Most circannual rhythms are less than 12 months long and photoperiod, acting through the hormone melatonin, serves to entrain the rhythm to a calendar year [1]. When deprived of the necessary environmental cues rhythms undergo a phase shift, resulting in the rhythm being dysynchronous with that of animals maintained in natural environments. In the present study, the animals housed indoors had a significantly greater α -TNF and IL-6 response to LPS stimulation in March compared to the animals in a natural environment. This may reflect an advancement of phase however the frequency of sampling in the study was insufficient to adequately assess a phase shift. Importantly, the presence of a seasonal immune rhythmicity in baboons housed indoors indicates that season must be controlled for in immune function studies regardless of where the animals are housed.

In this study factors other than photoperiod differed between the groups, including temperature, humidity, quality of light exposure and other environmental conditions. These factors may have contributed to seasonal differences in immune function. In addition, the animals housed in the outdoor corrals were in large groups of >50 animals, whereas the indoor animals were in small groups of 3 animals. Unfortunately, the facility constraints did not permit performing this study with equal size groups in the two housing conditions. Animal density can impact both the risk of pathogen transfer and social stress. Although the animals in study appeared healthy, the presence of subclinical infection cannot be excluded. In addition, animals in larger populations might have greater stress due to social hierarchy. Both stress and infectious disease are known to affect biorhythms as well as immune response [22]. To minimize the variability between the groups, whenever possible, stress provoking events were managed similarly. For example, for blood collection animals from both groups were moved into a novel cages and intramuscular ketamine was administered using squeezing as the method of restraint. Despite these confounding variables, the lack of major differences in immune stimulant-provoked cytokine release between the natural and controlled environmental groups suggests these factors were not important contributors to the seasonal variation of inflammatory response in the baboons of this study. Most importantly, the finding of circannual rhythmicity in immune response, regardless of its origin, indicates a need to control for season in experimental design.

One potential mechanism by which immune function could be seasonally regulated is in response to seasonal changes in anti-inflammatory hormone concentration. However, there was no correlation between PBMC cytokine response to stimulation and blood concentration of ACTH, α -MSH or cortisol. Furthermore, serum cortisol and α -MSH concentration was positively not negatively correlated with serum IL-6 concentration, suggesting anti-inflammatory hormones were released in response to the inflammatory state rather than the converse.

Understanding the pattern and underlying cause of disease seasonality is important if well designed preventative strategies are to be implemented. Seasonal outbreaks of infectious diseases have been well documented in people. Viral respiratory pathogens, including influenza and respiratory syncytial virus, are more common in nasopharyngeal swabs collected in the winter (January-March) compared to those collected in the summer [10]. Similarly, clinical outbreaks of seasonal influenza and respiratory syncytial virus occur most commonly between October and April [14]. Norovirus, the most commonly identified cause of infectious diarrhea of people in developed countries, is also more common in winter [18]. It is unclear if the seasonal pattern of disease outbreaks results from variation in exposure to pathogens, change in virulence of pathogens or seasonal alteration in host immunity. Cold or humid winter weather may affect viral particle replication or virulence directly [11, 20]. Increased social interaction, such as occurs when children return to school, may promote enhanced exposure to pathogens. The present study was not designed to assess the role of pathogen load in the baboons studied and there are no reports documenting seasonal patterns of disease outbreaks in captive baboons. So it remains unknown if the observed changes in inflammatory response were secondary to subclinical disease or a primary response to change in photoperiod.

The results of the present study confirm that season has a robust effect on inflammatory response in the reproductively non-seasonal baboon. Therefore it is important to consider season when designing a study in which cytokine response or markers of inflammation are to be assessed. Furthermore, studies performed in different seasons may have different outcomes, and caution should be exercised when combining data collected during different times of the year. Since it is common practice to move animals at the onset of experiments, often between natural and controlled environments, insight into the potential impact of

housing as an experimental variable is important. Results from this study found that housing did not abolish seasonal inflammatory response, however the design of the study precluded evaluation of potential shifts in the timing of seasonal fluctuations. Further studies are needed to quantify the magnitude of effect of photoperiod on phase shift of immune response in baboons. Due to the importance of the baboon as an animal model of infectious diseases and vaccine efficacy, further work is needed to determine the effects of season on adaptive immune responses, such as antibody production and cell-mediated immunity.

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Figure 1.

Serum concentration of c-reactive protein (Panel A) and interleukin-6 (Panel B) in young male baboons housed outdoors in natural light (gray bars) or indoors in a controlled environment of 12 hours light: 12 hours of dark (black bars). There was no effect of housing conditions on CRP concentration so the groups were combined to compare seasonal effects. Serum CRP was decreased in December as compared to in June ($P < 0.05$, Figure 1). Serum IL-6 concentration varied by season ($P < 0.001$) as well as by housing ($P < 0.001$), with no interaction between the two factors. In the outdoor baboons IL-6 concentration was significantly greater than that of the animals housed indoors at all timepoints ($P < 0.001$). In addition, IL-6 concentration in the outdoor animals was highest during the coldest time of year (December) and lowest in June, the warmest time collected. In the outdoor animals the IL-6 concentration was greatest in December while in the indoor animals IL-6 concentration was greatest in September (Figure 1).

Figure 2.

Serum cortisol, plasma ACTH and plasma alpha-melanocyte stimulating hormone (α -MSH) concentration. There was an interaction between the effect of housing and season on serum cortisol, therefore each housing group was analyzed separately. There was no effect of season on serum cortisol concentration in animals housed either in natural or controlled environment. Plasma ACTH (all months) and serum cortisol concentration (June, September, December) were greater in outdoor animals. There was no effect of housing or season on plasma α -MSH concentration.

Figure 3.

Increase in TNF- α (Panels A and C) or IL-6 (Panels B and C) release from peripheral blood mononuclear cell (PBMC) after stimulation with lipopolysaccharide (LPS, Panels A and B) or unmethylated CpG oligodeoxynucleotide (Panels C and D). A robust seasonal rhythm was present in baboons housed outside and those under a constant 12 hours light: 12 hours dark. LPS stimulation caused the greatest increase in cytokine release in June and September, and least response in December. In contrast, CpG had the greatest effect in June, with December showing the least response. Baboons housed in the controlled environment had a greater TNF- α release secondary to LPS treatment in March and a greater IL-6 release in September in response to CpG compared to those housed in a natural environment.

TABLE 1

Environmental Conditions of Baboons Housed Outdoors (Group 1)

	Daylength (hrs)	Mean Monthly Temp.(°C)
MAR	11.99	14.4
JUN	14.55	27.2
SEP	12.33	21.1
DEC	9.74	8.3