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Predictors and markers of resistance to neurotropic nematode infection in rodent host

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Abstract Parasite-mediated selection is currently believed to play an important role in life-history evolution. To assess how simple hematoserological and biochemical condition indices reflect host immunocompetence and infection resistance controlled laboratory experiments are required. We addressed these issues by infecting laboratory rats with the standard dose of embryonated eggs of a neurotropic nematode *Toxocara canis*. Urine baseline corticosterone concentrations, measured 1 week before infection, predicted the number of nematode larvae later recovered from host brains. Thus, this noninvasive clinical marker appeared useful for assessment of potential infection resistance. Rats who had accumulated high number of larvae in their brains and muscle had large spleens and high peripheral eosinophil counts 17 days postinfection. This finding is consistent with the concept that induction of eosinophilic Th2 type humoral immune response benefits the parasite rather than host. Hence, excessive peripheral eosinophilia and spleen enlargement are not markers of efficient antiparasite response in larval toxocariasis.

Introduction

Ecological immunology is a rapidly expanding field that examines the causes and consequences of variation in immune function in the context of evolution and ecology (Sheldon and Verhulst 1996; Zuk and Stoehr 2002). Immune function interacts with the general state of health of an organism and competes for the resources that can be allocated to other activities. Therefore, its assessment can potentially explain why individuals differ in their physiological condition and what are the fitness consequences of these differences. The use of hematoserological and biochemical condition indices (such as leukocyte differentials and steroid hormone levels) was often recommended for assessment of general condition and immune status of wild animals (e.g., Lochmiller and Dabbert 1993; Ots et al. 1998; Buchanan 2000; Wingfield and Sapolsky 2003) and the number of such studies is growing (reviewed by Buchanan 2000; Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; Wingfield and Sapolsky 2003). However, the question about how the individual condition should be measured and how it is linked to the state of individual's immune function (e.g., Braude et al. 1999; Buchanan 2000; Read and Allen 2000) or specific parasite resistance (Owens and Wilson 1999; Hanley and Stamps 2002; Ryder 2003; Adamo 2004) has raised some controversial issues. Examples include the questions about whether elevated leukocyte counts can be interpreted as an increased investment in immune system (Read and Allen 2000 vs Nunn et al. 2000); whether naturally occurring levels of stress hormones are immunosuppressive (Kurtz et al. 2000 vs Buchanan 2000); or whether large spleens reflect good immunocompetence (e.g., Møller and Erritzøe 2000) or ongoing infectious disease (e.g., Shutler et al. 1999; Smith and Hunt 2004). These questions cannot be answered properly without experimental manipulation of host infection status.

The aim of this study is (1) to elucidate whether easily measurable condition indices such as differential leukocyte counts and urine corticosterone levels can be used to predict host resistance to subsequent infection and (2) to find out which condition indices correlate with parasite resistance in infected hosts. As a model system, we use the infection of neurotropic nematode *Toxocara canis* in laboratory rat. *T. canis* is an ascarid parasite of carnivores that uses rodents as paratenic hosts. Subsequent to oral infection with embryonated eggs, infective larvae (L_3; Brunaska et al. 1995) of *T. canis* migrate through host

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tissues and contribute to pathology. Neurotropic larvae accumulate in the rodent's brain (where they can be quantified) 14 days after infection and presumably make the paratenic host vulnerable to predation for the final carnivorous host. Hence, this parasite should be suitable for detection of the fitness consequences of immune dysfunction in rodents because the fitness of the parasite depends on (1) its ability to survive the attacks of host's immune system and (2) its ability to debilitate host behavior and turn it to vulnerable prey (e.g., Cox and Holland 1998; Cuéllar et al. 2001). We expect that fitness-related variation in host condition will be reflected by differential resistance subsequent to infection with the standard dose of parasite and that markers of infection resistance potential can be elucidated by measuring correlations between condition indices and infection intensity. Further, we ask how different heavily infected individuals are from more resistant ones in terms of their health parameters measured in the course of disease and why. This issue also bears a certain applied meaning because larval toxocariasis (which constitutes a serious epidemiological problem in many countries) develops similarly in human and rodent hosts (e.g., Cuéllar et al. 2001). Yet, the functional importance of immune processes elicited by *T. canis* larvae in paratenic host appears enigmatic (e.g., Pritchard et al. 1997).

To assess the host condition, we used differential leukocyte counts known to reflect the state of activation of the immune system (e.g., Siegel 1985; Hoffmann-Goetz and Pedersen 1994; Dhabhar and McEwen 1997). In particular, we focused on the most numerous leukocyte types, namely, neutrophils, lymphocytes, and eosinophils. The typical response to infectious diseases, stress, and physical exhaustion in vertebrates is an increase in the concentration of circulating neutrophils and concomitant decrease in lymphocytes triggered by the release of glucocorticoid stress hormones. This process leads to increased neutrophil/lymphocyte ratio, which is a widely used index of stress and welfare in animals (e.g., Anderson et al. 1999). Eosinophils are prominent at sites of allergic reactions and with helminth and ectoparasite infections, hence, elevated number of peripheral eosinophils usually indicates a response to abnormal cells, parasites, or allergens.

To assess the role of hypothalamic-pituitary-adrenal (HPA) axis in infection resistance, we measured the urinary concentrations of corticosterone, which is the main stress hormone in rodents. The potential for application of urinary corticosterone as a diagnostic marker of individual immune status deserves attention because this assay provides a relatively easily obtainable noninvasive measure of host condition. Such markers are required, especially in research models of small mammals, which are technically difficult to bleed without causing major distress and also because of the problems related to rapid increase in circulating corticosterone levels due to capture or handling stress (e.g., Bamberg et al. 2001). Postmortem, we weighed the spleens, which are important immune and hemopoietic organs. Individual variation in spleen size was interpreted to reflect host immunocompetence; however, the direction of this relationship is not always clear (Smith and Hunt 2004).

Materials and methods

Animal maintenance

In total, 30 male outbred Wistar (Brl:Wist@Mol) rats were obtained from Taconic M & B, Denmark, at the age of 8 weeks on 25.09.2002. All rats were housed together in a separate room under standard vivarium conditions with 12:00-h light/12:00-h dark periods (lights off at 08:00) at a temperature of 21±2°C. Rats were housed in polypropylene cages (type 80-134G006) with 1,820 cm^2 floor area and external dimensions of 59.5 × 38 × 20 (high) cm, covered with wire lid and bedded with aspen shavings. Animals were housed in groups of five. Tap water and food pellets (R 70, Lactamin AB) were available ad libitum. Subsequent to the period of acclimatization and quarantine of 14 days, the researchers tamed the rats by handling them daily (15 min/cage) during 1 week. At the age of 16 weeks, rats were rehoused with new cage-mates and at the age of 18 weeks, 20 individuals were subjected to predator or control odor treatments for 2 h/day during six consecutive days. On the next day after last odor treatment, 20 rats were subjected to the sexual exhaustion experiment that lasted 6–7 h, while the rest of rats were deprived of food and water for 7 h (the results of this experiment will be reported elsewhere).

Urine collection, corticosterone and creatinine analysis

One day before the beginning of odor treatment, the first urine samples for corticosterone analysis were collected around 14:00 and 19:00 h. Third urine sample was collected 1 week later, immediately after termination of copulation experiment (for 20 rats) or after 7:00 h food and water removal (for ten rats) at about 19:00 h. For collection of urine, the animal was placed into the plastic box with a size of 20×30×20 (height) cm and urine was collected with a micropipette. If the animal had not urinated in the box during 5 min after copulation experiment, the urine was collected with a pipette on its ventral side during intraperitoneal injection of anestheticum for parasite inoculation experiment (most animals urinated during the injection). We were able to collect the samples of calc. 0.1–0.5 ml urine. Samples were frozen and stored at −18°C until analyzed. Urine corticosterone was measured using a ^{125}I radioimmunoassay kit (RS 490 11; IBL, Hamburg, Germany). Urinary creatinine was measured using a Jaffe method on a Cobas Integra 400 analyzer (Roche Diagnostics). Urinary corticosterone concentrations were expressed as nanogram corticosterone per milliliter creatinine to correct for differences in urine production rates between rats and across days (e.g., Brennan et al. 2000).

Blood sampling and analyses

Subsequent to urine collection after termination of the copulation experiment, animals were anesthetized with intraperitoneal injection of Diazepam (Nycomed Austria GmbH, Linz; 5 mg/ml) 1 ml/1 kg. About 10 min after injection, the tails of animals were warmed in an approximately 37°C-water bath to ensure adequate blood flow. Blood was collected from the lateral caudal vein. Blood smears for differential leukocyte counts were stained with Leishmann–Giemsa method and examined by an experienced observer to determine the proportions of lymphocytes, neutrophils and eosinophils per 100 leukocytes.

Experimental infection and estimation of parasite burden

Immediately after blood collection (when animals were still under anesthesia), rats were orally inoculated with calc. 10,000 *T. canis* embryonated eggs via stomach tube. This dose was used because it was shown to yield a detectable larval recovery from the brains in pilot experiment. Eggs were obtained from uteri of female intestinal adult worms, collected from 1-month-old German shepherd puppies (domestic pets) 2 days after deworming with fenbendazole (5 mg/kg body weight). The upper part of the uterus was removed, crushed on Petri dish, and eggs were collected into a test tube with tap water. Collected eggs were suspended in 0.1% formalin and poured into tissue culture flasks to a maximal depth of 5 mm and incubated at 25°C for approximately 1 month to embryonate. Embryonated eggs were stored at 4–8°C until use. Before experimental infections, egg suspension was washed with tap water three times. Seventeen days after infection, rats were killed by chloroform fumes in a 3-l exsiccator in a separate well-ventilated room, avoiding contact of personnel with chloroform fumes. This method of killing was used because of its rapid action and minimal stress caused to the animals, e.g., compared to intraperitoneal injections. Second blood, brain, spleen, and whole muscle tissue samples were collected. Spleens were weighed with a precision of 0.01 g with an electronic balance (Mettler Toledo AB-S). Larvae were recovered from brains and muscle tissue by HCl-pepsin digestion technique, modified after Kapel and Gamble (2000). Samples were digested in an artificial gastric fluid consisting of 10 ml 38% HCl and 10 g of pepsin powder (1:10,000) dissolved in 1 l tap water. Whole brains were gently crushed with tweezers, while muscle tissue was minced with a cleaver; muscle samples were divided to four portions for digestion. Thereafter, the samples were processed in a thermostat at 46°C with digestion a fluid on a magnetic stirrer for 2–3 h until the tissue was digested. Digested samples were left to settle for about 30 min, then the supernatant was removed, and new water was added. This procedure was repeated three to six times to remove tissue remnants. Cleaned sediment was transferred to a 50-ml tube, allowed to settle, and the larvae were counted from the remaining 5 ml of the sediment on Petri dishes under binocular microscope at 14×7 magnification.

Statistics

Relationships between immune parameters and infection intensity were examined by Spearman rank correlations. Sample sizes vary between different tests due to our inability to obtain sufficient amount of blood and urine from all rats. All tests are two-tailed. Significance level was set to 5%. Data are presented as mean±SD.

Results

Animals with high number of nematode larvae in their brains also had high larval counts recovered from the muscle tissue (r_s=0.59, P=0.0005, N=30). Urine corticosterone levels measured 1 week before inoculation correlated positively with the number of larvae recovered from brains (Fig. 1a,b). Urine corticosterone levels measured immediately before inoculation did not correlate with the infection intensity (Fig. 1c). We found no significant relationships between urine corticosterone levels and number of larvae recorded in muscle tissue (P=0.3–0.7, N=12) or between preinfection leukocyte proportions and infection intensity (P=0.2–0.6, N=30).

Animals with high urine corticosterone levels before blood sampling had less circulating lymphocytes and more neutrophils, and correspondingly, higher N/L ratios than animals with low corticosterone levels (Fig. 2). Eosinophil proportion did not correlate with urine corticosterone concentration (r_s=−0.04, P=0.9, N=12).

Proportion of eosinophils recorded on day 17 postinfection (when animals were killed) correlated positively with number of larvae recovered from brains (r_s=0.64, P=0.0007, N=24) and from muscle tissues (r_s=0.73, P=0.00005, N=24). Proportion of lymphocytes recorded on day 17 postinfection correlated negatively with larval counts in brain (r_s=−0.46, P=0.024, N=24) and muscle (r_s=−0.44, P=0.031, N=24). Neutrophil counts and N/L ratios measured at the same time did not correlate significantly with larval counts in brain or muscle (P=0.4−0.9).

Spleen size increased with increasing infection intensity in brain (r_s=0.50, P=0.005, N=30) and muscle (r_s=0.49, P=0.006, N=30). Rats who did not develop brain infection had lower percentage of circulating eosinophils on day 17 p.i. (7.3±5.3) than rats who developed brain infection (12.5±3.8, $t_{8,14}$=2.5, P=0.018). Rats with brain infection also had larger spleens (83.6±11 mg) than rats without larvae in their brains (73.8±7.0 mg, $t_{10,18}$=2.6, P=0.015). Similar differences occurred between eosinophil counts and spleen sizes of rats with and without detectable muscle infection (P=0.02−0.0003).

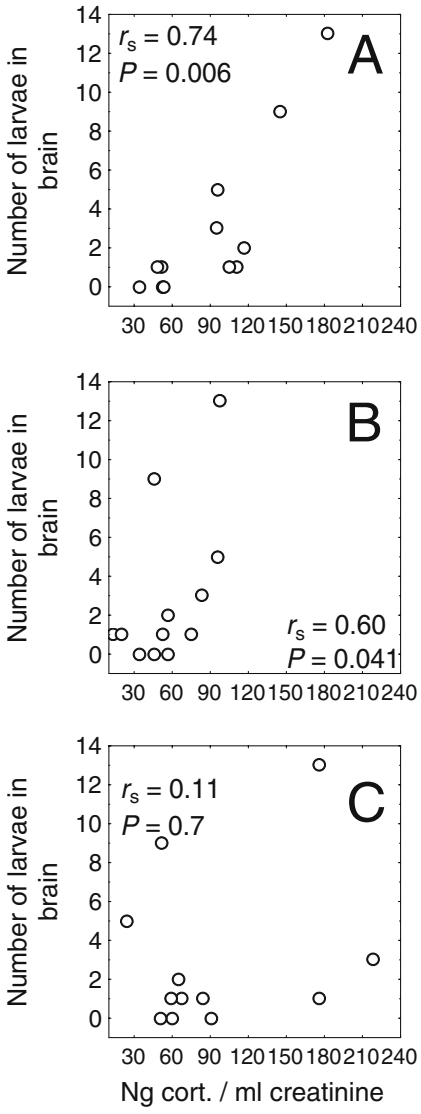

Fig. 1 Relationships between number of *T. canis* larvae recovered from brains 17 days postinfection and urine corticosterone concentration measured **a** around 14:00 h, 7 days before infection; **b** around 19:00 h, 7 days before infection; **c** around 19:00 h, immediately before infection. All data sets are based on the same 12 individuals

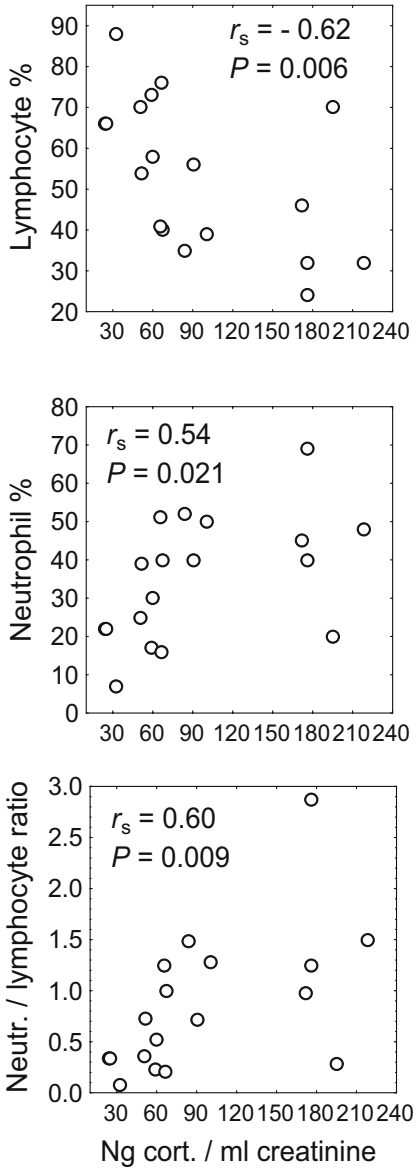

Fig. 2 Relationships between urine corticosterone concentration measured immediately before blood sampling and leukocyte differentials. All data sets are based the same 18 individuals

Discussion

Baseline urinary corticosterone concentrations measured 1 week before experimental infection significantly predicted the resistance of rats to neurotropic nematode infection in the brain. That glucocorticoids suppress immune responses and reduce inflammation was a conventional wisdom (e.g., Siegel 1985; Apanius 1998; Buchanan 2000). On the other hand, it was more difficult to find out whether (Buchanan 2000; Kurtz et al. 2000) and under which conditions (Barnard et al. 1996; Smith et al. 1996) are naturally occurring levels of stress hormones really immunosuppressive; and whether this extent of immune suppression has a real impact on individual fitness in terms of infection resistance (e.g., Owens and Wilson 1999; Hanley and Stamps 2002; Ryder 2003). This study gives a positive answer to the both questions, implying that between-individual differences in baseline hormone levels among animals reared under maximally standardized conditions are relevant in explaining the interindividual variation in subsequent infection resistance. This is an important finding because it means that a simple, noninvasively collectable hormonal marker contains information about the individual's potential to resist infection even 1 week after the hormone measurement. Urinary corticosterone levels in caged rodents were used for assessment of stress response (Brennan et al. 2000) or animal welfare (Augustsson et al. 2002) and this method was shown to be more appropriate for assessment of long-term aversive stimulation than measurement of blood glucocorticoid levels (Brennan et al. 2000; Bamberg et al. 2001). A need for such a remote method of sampling stress

hormones was recognized by animal ecologists (Buchanan 2000) and the results of the present study confirm its suitability for detection of biologically meaningful variation in corticosterone levels. Hence, this method should be potentially applicable for estimation of baseline immune status in small mammal models, such as voles or ground squirrels, which can be confined for a short period for urine collection. Further studies should also test for the relationship between immune function and fecal stress hormone levels, which are even more easily obtainable than urine samples. Preliminary studies in laboratory rats (Bamberg et al. 2001) and different wild mammals (reviewed by Lynch et al. 2003) have already indicated a great potential for such an approach.

As regards the optimal timing of urine collection, our results indicate that samples collected around 14:00 h were the most informative predictors of infection resistance potential (Fig. 1). This might relate to circadian rhythm of corticosterone levels, which peak in the beginning of the dark phase and subsequently decrease in nocturnally active animals (Retana-Márquez et al. 2003). Maximal increases of stress hormones in response to acute stressors in rats occur during the light period (reviewed by Retana-Márquez et al. 2003), suggesting that measurements taken in the dark period are more reflective of baseline values and less confounded by variation in handling stress or its perception by animals. Hence, if the baseline corticosterone levels are indicative of truly immunosuppressive chronic stress and such baseline levels are more easily detectable in the middle than in the end of the dark phase, then our result about the strongest correlation between urinary corticosterone and infection susceptibility is expected. In addition, we cannot exclude the possibility that urinary corticosterone levels collected around 19:00 h (Fig. 1b) were more affected by the previous handling stress than in samples collected earlier on the same day (Fig. 1a). Lack of correlation between infection intensity and urinary corticosterone collected immediately before experimental infection (Fig. 1c) could be again due to acute stress-induced variation in corticosterone secretion, as most of animals were involved in sexual exhaustion experiment 6−7 h before final urine collection. This might also (at least partly) explain why we failed to detect significant correlations between leukocytic immune parameters measured immediately before infection and parasite resistance. Given the relatively straight correlations between urine corticosterone and leukocyte proportions (Fig. 2), one might speculate that leukocyte differentials were primarily affected by HPA-axis activation due to acute stress, rather than being informative about baseline immune status of an animal. On the other hand, it should be kept in mind that our infection model involved a macroparasite and we might have discovered different relationships between leukocyte profiles and infection using bacterial, protozoan, or viral pathogens (see Hoffman-Goetz and Pedersen 1994; Barnard et al. 1996; Smith et al. 1996). Anyway, the strong relationships between urinary corticosterone and leukocyte profiles (Fig. 2) further emphasize the value of urinary corticosterone concentration as the measure of individual immune status.

What did our experiment reveal about markers of disease resistance in larval toxocariasis? Animals who had accumulated high number of larvae in their brains and muscle had large spleens and high peripheral eosinophil counts postmortem. These findings are important for studies examining intraspecific variation in spleen size or leukocyte differentials in the context of immunocompetence. For instance, it is sometimes assumed that large spleens indicate the host's ability to produce strong immune response (e.g., Møller and Erritzøe 2000; Hosken and O'Shea 2001; Acquarone et al. 2002; but see Shutler et al. 1999; Smith and Hunt 2004). Our results clearly show that at least in larval toxocariasis, large spleens do not indicate efficient parasite clearance ability because individuals who were least resistant to infection developed the largest immune defense organs. Similarly, although eosinophils are involved in immune responses against macroparasites, our study showed that high peripheral eosinophil counts do not indicate a superior ability to fight infections.

Why then were individuals with most active immune response most susceptible to the infection? Larval toxocariasis, like most helminth infections, induces Th2 type cytokine response that is responsible for IgE secretion and proliferation and activation of eosinophils (e.g., Ovington and Behm 1997). However, the question whether such a response serves primarily the interests of hosts or parasites in larval helminth infections is currently under debate (e.g., Pritchard et al. 1997; Meeusen and Balic 2000). Because the evidence that Th2-driven eosinophilic response can effectively damage the parasites is equivocal, it was suggested that cytokines secreted by Th2 lymphocytes may benefit the parasite rather than the host due to suppression of Th1 type inflammatory response (Allen and Maizels 1997; Pritchard et al. 1997; van der Kleij et al. 2002). The concept of regulatory balance between inflammatory and humoral immune response is based on differentiation of T-helper lymphocytes into two functional subsets with contrasting and cross-regulating cytokine profiles. Cross-regulation between these immune responses means that for instance, Th2 responses may exert anti-inflammatory action by negatively regulating Th1-cell-mediated immunity and vice versa (e.g., D'Ambrosio et al. 2000). It is important to note that glucocorticoid stress hormones in mice and rats also suppress Th1 responses and enhance Th2 cytokine secretion (see MacPhee et al. 2000). In this context, our results demonstrating a positive correlation between parasite infection success and peripheral eosinophilia and urinary corticosterone levels are consistent with the view that successful clearance of this parasite indeed requires effective Th1 type inflammatory response. It is also possible that positive correlation between spleen size and infection intensity is due to the expansion of the splenic B cell pool and associated lymphoid tissue (also see Kayes 1984) due to elevated Th2 response.

In conclusion, we have shown that measurement of urinary corticosterone levels in rat provides a relevant and biologically meaningful method for assessment of the host immune status. Excessive peripheral eosinophilia and spleen enlargement are not markers of efficient antiparasite response in larval toxocariasis, but rather result from successful host manipulation by parasite, leading to host's inability to develop adequate inflammatory response.

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