MHC Class II DRB Variability and Parasite Load in the Striped Mouse (Rhabdomys pumilio) in the Southern Kalahari

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Major histocompatibility complex (MHC) variability is believed to be maintained by pathogen-driven selection, mediated either through heterozygous advantage or frequency-dependent selection. However, empirical support for these hypotheses under natural conditions is rare. In this study, we investigated the genetic constitution of the functionally important MHC class II gene (DRB exon 2) and the parasite load in a population of the striped mouse (Rhabdomys pumilio) in the Southern Kalahari. Fifty-eight individuals were genetically examined and the endoparasite load was quantified by counting fecal helminth eggs by using a modified McMaster technique. Thirty-four animals (58.6%) were infected. We identified 20 different MHC alleles with high levels of sequence divergence between alleles. Particularly, the antigen-binding sites revealed a significantly higher rate of nonsynonymous substitutions (dN) than synonymous substitutions (dS), giving strong evidence of balancing selection. Heterozygosity did influence the infection status (being infected or not) and the individual fecal egg count (FEC) value with significantly higher values observed in homozygous individuals. Furthermore, a positive relationship was found between specific alleles and parasite load. The allele Rhpu-DRB*1 significantly occurred more frequently in infected individuals and in individuals with high FEC values (high parasite load). Individuals with the allele Rhpu-DRB*1 had a 1.5-fold higher chance of being infected than individuals without this allele (odds ratio test, P < 0.05). Contrarily, the allele Rhpu-DRB*8 significantly occurred more frequent in individuals with low FEC values. Our results support the hypotheses that MHC polymorphism in R. pumilio is maintained through pathogen-driven selection acting by both heterozygosity advantage and frequency-dependent selection.

Introduction

Within the vertebrate’s genome the prime candidate for resistance genes is the major histocompatibility complex (MHC) which contains some of the most polymorphic functional loci in vertebrates (Hedrick 1994). The MHC encodes cell-surface glycoproteins that bind antigens derived from pathogens or parasites and present them to T lymphocytes to initiate the immune response. The variability of the MHC molecules is correlated with the diversity of the T-lymphocyte receptors, which in turn determine the disease and parasite resistance of an organism (Klein 1986). High levels of variability are manifest at the molecular level by an increased ratio of nonsynonymous over synonymous substitutions at the functionally important antigen-binding sites (ABS) and is considered as a clear indication for selection processes (Hughes and Nei 1988, 1989; Bergström and Gyllensten 1995; reviewed in Jeffery and Bangham 2000). Thus, the two most debated selection hypotheses explaining the high MHC polymorphism refer to pathogen-based mechanisms (Hedrick and Kim 2000): the heterozygote advantage hypothesis (overdominant selection) and the rare allele advantage hypothesis (negative frequency-dependent selection) (Clarke and Kirby 1966; Doherty and Zinkernagel 1975).

The heterozygote advantage hypothesis presumes that heterozygous individuals are favored because they possess more different alleles than homozygous individuals and are therefore able to recognize a broader spectrum of pathogens. This was verified in humans with regard to hepatitis B and human immunodeficiency virus infections (Thursz et al. 1997; Carrington et al. 1999) and in mice with salmonella infections (Penn, Damjanovich, and Potts 2002). However, so far there is only little evidence from population surveys and experimental infections to support this hypothesis (Penn and Potts 1999; Arkush et al. 2002).

The rare allele advantage hypothesis assumes that MHC diversity is maintained through frequency-dependent coevolutionary processes between hosts and parasites (Takahata and Nei 1990). The most resistant allele will be favored and spread through the population. However, it will not go into fixation because when the resistant allele becomes common, this increases selection on parasites to evade the recognition by this common allele. In the end this leads to an increased variability within a population (reviewed in Jeffery and Bangham 2000). There are several studies which showed an association between specific MHC alleles and pathogen resistance (e.g., Langefors et al. 2001; Lohm et al. 2002; Harf and Sommer 2005; Meyer-Lucht and Sommer 2005; Schad, Ganzhorn, and Sommer 2005).

The MHC system is one of the few genetic systems where balancing selection has been revealed in humans and in murid model organisms under laboratory conditions, but studies in free-ranging wild animal populations are still rare (Bernatchez and Landry 2003).

In this study, we used the striped mouse (Rhabdomys pumilio), a widely distributed diurnal rodent in Africa, as a model organism to examine associations between MHC genotypes and parasite load under natural conditions. The specific objectives of this study were (1) to investigate both MHC constitution and parasite burden in R. pumilio, (2) to examine the importance of MHC genotypes for resistance to parasites, and (3) to investigate if there are indications for balancing selections responsible for high polymorphism in the MHC. We have focused on exon 2 of the MHC class II gene DRB, which encodes the major part of the ABS and has been described to be the most polymorphic part in many class II genes (Brown et al. 1993; Ohta 1998; Hughes 1999).
Materials and Methods

Study Areas and Sample Collection

The study took place in the Southern Kalahari Desert, 30 km south of the Kgalagadi Transfrontier Park in South Africa. The study area lies in the Shrubby Kalahari Dune Bushveld, which is a subdivision of the Savanna biome (Low and Rebelo 1996). The mean annual rainfall ranges from 150–350 mm. Animals were caught in June 2002 with Sherman® traps by using standardized grids and were individually marked. Fifty-eight small ear tissue samples were collected and fixed in 70% ethanol for genetic analyses. Trapped animals were kept overnight, and individual fecal samples were collected from each trap for later investigations of the parasite load. Traps were cleaned before reuse. Fecal samples were preserved in 5% formaldehyde to investigate the parasite burden. Afterwards, the animals were released at their respective trapping sites.

Molecular Techniques

We examined variation of a highly polymorphic 171-bp fragment of exon 2 of the MHC class II DRB gene which includes the functionally important antigen-binding and recognition sites. To identify primers we performed an extensive Blast search (http://www.ncbi.nlm.nih.gov/blast/) was carried out, and DRB sequences from a wide range of animal species from different phylogenetic radiations were aligned. Primers were designed in a way that they bind to conserved parts of DRB exon 2 in many taxa. Polymerase chain reaction (PCR) amplification of partial DRB exon 2 was carried out using primers JS1 and JS2 as described in Schad, Sommer, and Ganzhorn (2004). The primers JS1 and JS2 successfully amplified this locus in different lemur and rodent species, e.g., Microcebus murinus (Schad, Sommer, and Ganzhorn 2004; Schad, Ganzhorn, and Sommer 2005), Gerbillurus paeba (Harf and Sommer 2005), Leopoldamys sabanus (Lenz, T. and Sommer, S. unpublished data), Rattus rattus (Hingston, M., Schmidt, D., and Sommer, S., unpublished data).

To identify allelic diversity, all individuals were subjected to single-strand conformation polymorphism analyses (Orita et al. 1989). PCR products were loaded on 15% polyacrylamide gels following the manufacturer’s protocol (ETC Elektrophoreseotechnik, Kirchentellinsfurt, Germany) and run on a horizontal cooling electrophoresis system (Amersham Pharmacia Biotech, Freiburg, Germany). The configuration of the bands was visualized by silver staining. Samples were rearranged and run again according to assessed similarities. All identified alleles were sequenced bidirectionally. Therefore, at least three examples of each allele were excised from the gel, dissolved in 1 x TBE buffer, and reamplified under the same PCR conditions mentioned above. Cycle sequencing of the PCR products was performed using a dye-terminator sequencing kit (Applied Biosystems, Foster City, Calif.) and then analyzed by gel electrophoresis with an Applied Biosystems automated sequencer model 3100, following the manufacturer’s instructions. Details on the molecular techniques are outlined in Sommer and Tichy (1999), Sommer, Schwab, and Ganzhorn (2002), Sommer (2003, 2005).

Parasitic Screening

For the identification and quantification of parasite eggs, we applied the noninvasive fecal egg count (FEC) method (Dunn 1978). FEC is a noninvasive and an appropriate tool to estimate the dimension of nematode infections (Sousbys 1982) and was utilized in a number of recent investigations (e.g., Cassinello, Gomendio, and Roldan 2001; Irvine et al. 2001; Seiwright et al. 2004). All 58 fecal samples were screened for helminth eggs by using a modification of the McMaster flotation technique (Gordon and Whitlock 1939) by using a flotation-dilution of potassium iodide with a specific weight of 1.5 g/ml (Meyer-Lucht and Sommer 2005). Two chambers of McMaster were counted per sample. Helminths were assigned to morphotypes according to size and morphological characteristics.

We calculated individual FEC values as eggs per gram feces (EPG) and the number of different nematode morphotype infections per individual.

Statistical Treatment

Nucleotide sequences were aligned using GeneDoc version 2.6 (K. B. Nicholas and H. B. Nicholas 1997). Rates of nonsynonymous (dN) and synonymous base pair substitutions (dS) were calculated using the program MEGA (Kumar et al. 2001) according to a model originally described by Nei and Gojobori (1986). The Jukes and Cantor (1969) correction was applied for multiple substitutions. For parasite analyses, EPG values were log transformed to improve normality. Odds ratio tests were conducted to assess the relative risk of being infected. It is a common test in epidemiological studies to evaluate the exposition of individuals carrying a risk factor. The ratio of the odds of an event occurring in one group is compared to the odds of it occurring in another group by using a 2 x 2 cross-classification table (Sachs 1992). All additional statistical tests were performed by using SPSS version 9.0. Calculations are two-tailed and based on a significance level of α = 0.05. Bonferroni-corrected significance levels and the Tukey post hoc tests were used for multiple comparisons and calculations of pairwise differences (Rice 1989; Sachs 1992).

Results

MHC Variability

The 58 genetically examined individuals showed high levels of variability in the MHC DRB gene exon 2. We found 20 different alleles which were labeled following the nomenclature of Klein et al. (1990) from Rhu-DRB*1 to Rhu-DRB*20, according to their frequency in the study population. A Blast search indicated the amplification of the correct locus. The sequences were submitted to GenBank (accession numbers AY928312 - AY928331) (supplementary figure). Two Rhu-DRB alleles (Rhu-DRB*1 and Rhu-DRB*2) were common with a prevalence of more than 10%, whereas the remaining 18 Rhu-DRB alleles were rarely represented in the sample (<0.1%, see supplementary figure). In the 20 different alleles, 43 (25.1%) of 171 nucleotide positions were variable. The alleles differed between 1 and 20 nucleotide positions ($\bar{x}$ = 11.45, standard error [SE] = 5.48). Only 3 (7.0%)
of 43 variable nucleotides were synonymous substitutions. Within these neutral substitutions, changes in the nucleotide sequence occurred but did not lead to an alteration of the amino acid. The nucleotide sequences were transformed into 20 different amino acids. Neither start codons nor stop codons were found. Within 57 amino acid positions, 23 (40.4%) have been detected as variable. Pairwise comparisons of amino acid sequences differed by 1 to 14 positions.

According to Brown et al. (1988, 1993) 15 of the 57 amino acids are involved in the functionally important ABS. All the other 42 positions belong to the non–antigen-binding sites (non-ABS). In the ABS, 12 (80.0%) out of 15 amino acids were variable. In contrast, only 11 (26.2%) of the 42 amino acids were polymorphic in the non-ABS. Nine out of 11 of these variable non-ABS sites were placed right next to an ABS.

In the ABS, the rate of nonsynonymous substitutions \( (d_N) \) was 1.59 times higher than the rate of synonymous substitutions \( (d_S) \) (table 1; \( t = -6.7, P < 0.001 \)). Within the non-ABS the rate of nonsynonymous substitutions was also significantly higher than the rate of synonymous substitutions \( (t = -9.3, P < 0.001) \), but both the nonsynonymous and the synonymous substitution rate was close to zero. All in all, nonsynonymous substitutions were found significantly more frequently in the ABS than in the non-ABS \( (t = 21.8, P < 0.001) \).

Parasite Load

The investigation of the 58 feces samples of R. pumilio revealed eight different nematode morphotypes. No cestodes and trematodes were found. Thirty-four animals (58.6%) were infected. Infected mice revealed between one (65%), two (29%), and three (6%) different nematode morphotypes. The two most abundant nematode morphotypes appeared in 50% and 38% of the animals, respectively.

Parasite Load and MHC Variability

In order to investigate the importance of the individual MHC genotype for resistance to parasites and possible selection mechanisms, associations of either heterozygosity (heterozygous advantage) or specific alleles (frequency-dependent selection) with nematode load were tested. Animals with a homozygote MHC genotype belonged significantly more often to the group of infected individuals than individuals with a heterozygote MHC (fig. 1; \( \chi^2 = 3.82, P = 0.05 \)). Whereas 80% of the homozygous individuals were infected, only 51% of the heterozygous animals carried nematode eggs. In addition, the parasite burden was significantly higher in homozygous animals, which showed a higher number of EPG than in animals with a heterozygote MHC genotype (fig. 2; \( t = -2.18, P = 0.03 \)). Furthermore, a positive relationship was found between specific alleles and parasite load. The allele Rhpu-DRB*1 significantly occurred more frequently in infected individuals \( (\chi^2 = 4.75, P = 0.03, \text{Bonferroni not significant}) \). Individuals with the allele Rhpu-DRB*1 had a 1.5-fold higher chance of being infected than individuals without this allele \( (\text{odds ratio test: } P < 0.05) \) (Sachs 1992). Also, the FEC values differed with respect to the DRB allele configuration. Individuals carrying Rhpu-DRB*1 exposed significant higher FECs than individuals

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### Table 1

<table>
<thead>
<tr>
<th>Position</th>
<th>N</th>
<th>( d_N )</th>
<th>( d_S )</th>
<th>( d_N/d_S )</th>
<th>( P )</th>
</tr>
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<tr>
<td>ABS</td>
<td>15</td>
<td>0.27 ± 0.07</td>
<td>0.17 ± 0.09</td>
<td>1.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-ABS</td>
<td>42</td>
<td>0.05 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>1.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>All</td>
<td>57</td>
<td>0.10 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>1.67</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Note.**—\( N \) is the number of codons in each category. \( P \) is the probability that \( d_N \) and \( d_S \) are different using a \( t \)-test.

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**Fig. 1.**—Number of infected (black bars, \( N = 34 \)) and noninfected mice (white bars, \( N = 24 \)) in heterozygous and homozygous individuals.

**Fig. 2.**—Fecal egg count (FEC) values of heterozygous (\( N = 43 \)) and homozygous (\( N = 15 \)) individuals.
without ($t = 2.38; P = 0.02$, post hoc tests: not significant; fig. 4). Contrarily, the allele Rhpu-DRB*8 occurred significantly more frequently in individuals with low FEC values ($t = 2.49, P = 0.04$, post hoc tests: not significant; fig. 4).

**Discussion**

We investigated the importance of MHC genotypes for resistance to parasites and the selective mechanisms which are acting on the MHC in the presence of parasites in 58 individuals of the striped mouse (*R. pumilio*) living in the Southern Kalahari under natural conditions. Twenty different MHC class II DRB exon 2 alleles were identified. In our *R. pumilio* study, no individual had more than two alleles suggesting that only one DRB locus was amplified. The DRB alleles showed high levels of sequence divergence for an intraspecific comparison and all alleles had a unique amino acid sequence.

Polymorphism was highest in the functionally important antigen recognition and binding sites. In these positions, significantly more nonsynonymous than synonymous substitutions were found. Both, the nonsynonymous and the synonymous substitution rate was close to zero in the non-ABS. This is considered as a clear indication for positive selection (Hughes and Nei 1988, 1989) and characteristic for proteins with antigen-presenting function (e.g., Seddon and Ellbergren 2002; Bernatchez and Landry 2003; Schad, Sommer, and Ganzhorn 2004).

Because the MHC plays a major role in the immune system of vertebrates, pathogen-driven selection processes are thought to be involved in the maintenance of diversity at MHC loci (Parham and Ohta 1996; Jeffery and Bangham 2000; Penn 2002; Bernatchez and Landry 2003). A few studies revealed an association between MHC and parasite load (Langefor et al. 2001; Arkush et al. 2002; Lohm et al. 2002; Wegner, Reusch, and Kalbe 2003; Harf and Sommer 2005; Meyer-Lucht and Sommer 2005, Schad, Ganzhorn, and Sommer 2005). However, studies conducted in wild vertebrate populations are still rare.

In this study, heterozygosity did influence the infection status (being infected or not). A significant high proportion of infected *R. pumilio* were homozygous. Furthermore, the individual FEC value was significantly higher in homozygote mice than in heterozygous ones. A bias in the typing of homozygous individual due to the presence of nonamplifying alleles (mutation in the primer-binding site) which would cause heterozygous individuals to be counted as homozygous is unlikely. Primers were tested in a number of species and proved to be very robust. Our results are in accord with the **heterozygote advantage hypothesis** which assumes that heterozygous individuals are able to recognize a broader array of pathogens. So far there is only little evidence in literature that heterozygous individuals may have an advantage compared to homozygous individuals (Carrington et al. 1999; Coltman et al. 1999; Penn, Damjanovich, and Potts 2002). Associations between MHC heterozygosity and infectious diseases in free-ranging animals under natural conditions

![Fig. 3.—Frequency of Rhpu-DRB alleles in infected (black bars) and not infected (white bars) individuals. Alleles with low prevalence (>5 individuals) are not displayed. $P < 0.05$.](image1)

![Fig. 4.—Fecal egg count (FEC) values with respect to the specific alleles. Alleles with low prevalence (>5 individuals) are not displayed. $P < 0.05$.](image2)
have been found in Chinook salmon (*Oncorhynchus tsawytscha*, Arkush et al. 2002) and in Gila topminnow (*Poeciliopsis occidentalis occidentalis*, Hedrick, Kim, and Parker 2001).

Furthermore, a positive relationship was found between specific alleles and parasite load. The allele *Rhpu-DRB*1 significantly occurred more frequently in infected individuals and in individuals with high FEC values (high parasite load). Individuals with the allele *Rhpu-DRB*1 had a 1.5-fold higher chance of being infected than individuals without this allele (odds ratio test, \( P < 0.05 \)). Contrarily, the allele *Rhpu-DRB*8 was significantly more frequent in individuals with low FEC values. In this respect, it is interesting to note that it is the most common allele *Rhpu-DRB*1 (allele frequency: 0.22) which is associated with high parasite load, whereas the rare allele *Rhpu-DRB*8 (allele frequency: 0.05) is associated with low parasite load—as might be predicted under negative frequency–dependent selection. Our study adds to the growing body of studies which support the rare allele advantage hypothesis (e.g., Potts and Wakeland 1990; Slade and McCallum 1992; Quinnell et al. 2003; Harf and Sommer 2005; Meyer-Lucht and Sommer 2005; Schad, Ganzhorn, and Sommer 2005).

This hypothesis assumes that an individual with a rare MHC allele might respond better to a new parasite variant and cause an advantage to the host. An allele that provides better immunity against parasites will increase in frequency within a population (Parham and Ohta 1996). But after a while parasites will get adopted to those specific alleles and may become a disadvantage for the host. This might have happened with the *Rhpu-DRB*1 allele which was associated with high parasite load. Contrarily, *Rhpu-DRB*8 might be an example of a new emerging allele still associated with high parasite resistance.

We are aware that this study is based on a relatively small sample size which might have influenced the results and prevented more in-depth statistical analyses. The results derived from a small but functionally important part of the MHC. Increasing sequence information of the MHC in a number of species from different phylogenetic radiation will help develop universal primers to amplify larger parts of the MHC.

To conclude, our results support the hypotheses that MHC polymorphism in *R. pumilio* is maintained through pathogen-driven selection acting by both heterozygosity advantage and frequency-dependent selection. Right now it is not quite clear whether heterozygote advantage and frequency-dependent selection hypothesis is most important for balancing selection (Hedrick 2002). It is conceivable that a rare allele may have a high fitness and at the same time a constant advantage for heterozygotes. Both hypotheses may be in accord with each other and are not mutually exclusive. For further investigation of the selection hypotheses, allele frequencies and parasite burden need to be followed through time to see whether allele frequencies change in a cycling pattern.

**Supplementary Material**

Supplementary data are available at *Molecular Biology and Evolution* online (www.mbe.oupjournals.org).

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**Literature Cited**


