THE ROYAL SOCIETY

Received 4 December 2003
Accepted 1 March 2004
Published online 16 June 2004

Major histocompatibility complex controls the trajectory but not host-specific adaptation during virulence evolution of the pathogenic fungus *Cryptococcus neoformans*

Erin E. McClelland[1*], Frederick R. Adler[1,3], Donald L. Granger[2] and Wayne K. Potts[1]

[1]*Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112, USA*
[2]*Division of Infectious Diseases, University of Utah and Veterans Affair's Medical Center, 30 North 1900 East, Salt Lake City, UT 84132, USA*
[3]*Department of Mathematics, University of Utah, 155 South 1400 East, Salt Lake City, UT 84112, USA*

Genes of the major histocompatibility complex (MHC) play a critical role in immune recognition and are the most genetically diverse loci known. One hypothesis to explain this diversity postulates that pathogens adapt to common MHC haplotypes and thus favour selection of new or rare alleles. To determine whether the pathogenic yeast *Cryptococcus neoformans* adapts to MHC-dependent immune responses, it was serially passaged in two independent replicate lines of five B10 MHC-congenic strains and Balb/c mice. All passaged lines increased in virulence as measured by reduced host survival. MHC influenced the rate (trajectory) of virulence increase during passages as measured by significant differences in mortality rate ($p < 0.001$). However, when the post-passage strains were tested, no MHC differences in mortality rate remained and only minor differences in titres were observed. Also contrary to expectations, increased virulence in three lines passaged in B10 mice had a larger effect in Balb/c mice, and the evolution of virulence in lines passaged in alternating hosts was not retarded. To our knowledge, these data represent the first experimental test of MHC-specific adaptation in a non-viral pathogen. The failure to observe MHC effects despite dramatically increased virulence and host-genotype-specific adaptation to non-MHC genes suggests that escape of MHC-dependent immune recognition may be difficult for pathogens with unlimited epitopes or that other virulence factors can swamp MHC effects.

Keywords: major histocompatibility complex; emerging pathogens; pathogen adaptation; *Cryptococcus neoformans*

1. INTRODUCTION

The proteins encoded by major histocompatibility complex (MHC) genes present antigenic peptides for immune recognition by T cells. Different MHC alleles confer resistance and susceptibility to a wide variety of pathogens and autoimmune diseases (Baum & Staines 1997; Thursz *et al.* 1997; Carrington & Bontrop 2002; Hildesheim & Wang 2002). Pathogens have numerous ways to escape MHC-dependent immune recognition (Potts & Slev 1995), which may provide a selective disadvantage to common alleles and an advantage to rare alleles, that is, negative frequency-dependent selection. This mechanism could explain the extreme diversity of MHC genes (Borghans *et al.* 2004) and is referred to here as the pathogen-adaptation hypothesis (Haldane 1949; Bodmer 1972; Takahata & Nei 1990).

Serial passage of pathogens offers an experimental technique for testing the pathogen-adaptation hypothesis (Messenger *et al.* 1999). Serial-passage experiments with many different pathogens have demonstrated that most pathogens increase in virulence when passaged in a new host species and lose virulence to their 'old' host species (Ebert 1998). This trade-off between increased virulence in the host of passage and attenuation of virulence in other hosts is also thought to occur at the genotypic level within host species and is a critical assumption in many models of host–parasite coevolution (Beltman *et al.* 2001; Woolhouse *et al.* 2002), the maintenance of sex and genetic diversity (Hamilton 1982; Dybdahl & Lively 1998) and the evolution of virulence (Regoes *et al.* 2000). This trade-off has been demonstrated in phage infections of different species of bacteria (Crill *et al.* 2000) and in a mouse–nematode model (Dobson & Owen 1977), but opposite patterns have been reported in a mouse–malaria model (Mackinnon *et al.* 2002).

Pathogen escape of MHC-mediated immune recognition is the best-studied genotype-specific adaptation. Studies have used a variety of different viruses: lymphocytic choriomeningitis virus (Pircher *et al.* 1990), hepatitis B virus (Bertoletti *et al.* 1994), Epstein–Barr virus (de Campos Lima *et al.* 1994), mouse hepatitis virus (Pewe *et al.* 1996), human immunodeficiency virus (HIV) (McMichael & Phillips 1997) and simian immunodeficiency virus (SIV) (Evans *et al.* 1999; Allen *et al.* 2000). These viruses were used either because of the high error rate found during replication (Preston *et al.* 1988; Roberts *et al.* 1988) or because they accumulate mutational

*Author and address for correspondence: Department of Medicine, Albert Einstein College of Medicine, 702 Golding, 1300 Morris Park Avenue, Bronx, NY 10461, USA (mcclella@aecom.yu.edu).

variants during chronic infections (Hosono *et al.* 1995). In all of these studies, escape variants were detected in MHC-presented epitopes, but only the SIV studies represent an experimental confirmation of MHC-dependent escape in a fully immunocompetent host. Additionally, none of these studies passaged viral variants through one host and then tested virulence in a different host to test for host-specific adaptation.

Escape from MHC-dependent immunity, to our knowledge, has not been previously tested with non-viral pathogens. We did so by conducting serial passages with *Cryptococcus neoformans* in five MHC-congenic strains of B10 mice and one strain of Balb/c mice. *Cryptococcus neoformans* is a facultative pathogenic yeast that causes disease primarily in immunocompromised humans. However, occasional cases of disease occur in immunocompetent humans and in a broad range of domestic animals and plants (Meadows *et al*. 1993; Campisi *et al*. 2003; O'Toole *et al.* 2003). This pathogen was chosen for the following reasons: (i) it is a chronic pathogen, which allows increased opportunity for immunoselection during each passage; (ii) it requires both opsonizing antibodies and cell-mediated immunity for protection; (iii) it is a well-studied non-viral pathogen that is a well-established model in mice and whose genome sequence allows detailed follow-up studies; and (iv) it is simple to work with, allowing easy quantification of infection titres and numerous replicates in a variety of mouse strains. Additionally, MHC-dependent susceptibility to *C. neoformans* has been established (McClelland *et al.* 2003*b*).

Serial-passage experiments predict that selection will favour pathogen genotypes with higher replication rates because they will be transmitted preferentially in the passage inoculum. If pathogens adapt to MHC-dependent immune responses, we predict the following results from these serial-passage experiments: (i) the pathogen will increase in virulence with passage (Ebert 1998); (ii) virulence increase will be greatest in the host of passage (Apanius *et al.* 1997); (iii) pathogen virulence will attenuate in genotypes different from host-of-passage genotypes (Ebert 1998); and (iv) if two or more MHC genotypes are alternated during passage, the pathogen will have retarded adaptation as measured by a smaller increase in virulence (Ebert 1998; Carrington *et al.* 1999). Although one of these predictions was fulfilled, surprisingly most were not.

2. MATERIAL AND METHODS

(a) *Mice*

MHC-congenic mice (C57BL/10SnJ-$H2^b$, B10.D2-$H2^d$, B10.M-$H2^f$, B10.BR-$H2^k$, B10.Q-$H2^q$) and Balb/c mice were obtained from Jackson Laboratories and bred thereafter under specific-pathogen-free conditions. The inclusion of Balb/c mice allowed comparisons between host strains that have identical MHC but differ at other loci that may affect *C. neoformans* resistance. All animals used for infections were either F_2 segregants or first-generation progeny of F_2 segregants. MHC F_2 segregants were created by intercrossing the F_1 heterozygotes (*b*/*q*, *d*/*q*, *d*/*k*, *f*/*k*) to randomize any genetic mutations that might have become differentially fixed in the backgrounds of these strains of mice. Mutation accumulation among different congenic strains creates genetic variation that can confound conclusions regarding strain differences. This problem becomes incrementally greater with each generation and some of these MHC B10 congenic strains have been separated for over 60 years (Carroll & Potts 2001 and references therein). Generating F_2 segregants randomizes any background mutations while leaving the MHC region intact (owing to high linkage disequilibrium) and therefore solves this problem in a much simpler way than re-deriving the lines (Gerlai 1996; Wolfer & Lipp 2000; Carroll & Potts 2001). Mice were MHC-genotyped at two microsatellite loci within the MHC region (a tetranucleotide repeat (Saha & Cullen 1986) and d17Mit34 (Blake *et al.* 2002)) using PCR for DNA amplification and denaturing gel electrophoresis for scoring band size. All animal use complied with federal regulations and the University of Utah's Institutional Animal Care and Use Committee guidelines.

(b) *Pathogen*

The human-isolated strain of *C. neoformans* H99 (Perfect *et al.* 1980) was used to initiate all passage infections in mice. After the first passage in mice, all experimental lines of *C. neoformans* were delineated by the MHC-congenic strain of mice from which they were isolated and were assumed to be descendants of the initial strain of H99. For example, $H99^B$ refers to the experimental line of *C. neoformans* that was passaged in $H2^b$ mice.

(c) *Infection and passages*

For the first infection, five adult mice per strain (more than six weeks old) were infected via intraperitoneal (i.p.) injection with $(2.5 \times 10^7$ colony forming units (CFU) ml^{-1} of strain H99 (Perfect *et al.* 1980). Mice were then killed 39 days after infection. This end date was chosen to allow the infection to proceed long enough to see a MHC-based effect but not long enough to risk death of the mice. At the time of sacrifice for the first infection, there were no obvious signs of clinical disease, as measured by weight loss and neurological symptoms, though some of the mice had ruffled fur (an indication of infection or disease) and many had lost 5% of their body weight.

Initially, the spleen, liver and brain were collected to determine which organ had the most consistent loads of *C. neoformans*. Brain loads were highly variable during this chronic infection whereas spleen and liver loads were highly correlated. The liver was chosen as the optimal organ for collection because it had the most consistent loads and was a site of primary infection with an i.p. injection.

Five different strains of B10 MHC-congenic mice and the Balb/c strain were used for passages (see § 2a). In addition, two alternating passages were conducted. In one, *d*/*d* and *q*/*q* MHC homozygote mice were alternately infected. For the other, *b*/*q*, *d*/*q* and *f*/*q* MHC heterozygote mice were alternately infected. Each strain was independently infected and passaged in duplicate (A and B) lines, except for the alternating passages.

Two mice per independent duplicate line were infected i.p. with 1 ml of liver homogenate in 10 ml of phosphate-buffered saline (PBS) and weighed on alternate days to assay host condition. Occasionally, when mice were scarce, only one mouse was infected per independent duplicate line. Consequently, mice were infected with unknown titres, which were later determined by counting colonies from plated liver homogenate. The between-passage titre dose was not controlled to eliminate the necessity of *in vitro* expansion, therefore removing the possibility of artificial selection associated with *in vitro* culture conditions. Mice were killed when phenotypically moribund (greater than

10% weight loss, ruffled fur, hunched posture, lack of movement, slow and heavy breathing and glazed eyes) and the mouse with the highest liver load was used to initiate the next passage. Since the mice were killed when phenotypically moribund, a condition associated with imminent death in previous studies, we use the term 'mortality' throughout the paper even though this is not technically mortality, but rather, an estimate of mortality. Eight successive passages were carried out for a total of nine infections.

To determine whether increased replication rate was correlated with increased virulence *in vivo*, we separately infected *q*/*q* MHC-congenic mice with the original dose (2.5×10^7 CFU ml^{-1}) of the pre-passaged H99 as well as a post-passaged line ($H99^{Q.B}$). All mice were killed on day 6 since this was the day when mice that were infected with $H99^{Q.B}$ began to die. Liver and brain titres were then determined.

Because there were marked differences in the mean day of death between passaged lines, the overall time in each specific MHC host differed (ranging from 125 to 327 days, depending on the line). Based on a generation time of 2–4 h *in vitro* (data not shown), we assume that each passaged line of *C. neoformans* spent at least 500–1000 generations in their specific host of passage, which should allow substantial opportunity for adaptation.

(d) Cryptococcus neoformans *titres*

Cryptococcus neoformans titres were determined from platings of the homogenized livers and brains. Brains were collected routinely after the third to fifth passage, depending on the passaged line. Livers and brains were collected and then homogenized in 10 ml of PBS. Then, 10 µl of the homogenate was diluted in 90 µl of PBS in serial dilutions to 1×10^{-5}. For each dilution, 10 µl was plated on yeast extract peptone glucose agar plates containing 10^3 U ml^{-1} penicillin, 1 mg ml^{-1} of gentamicin and 0.1 mg ml^{-1} of chloramphenicol. Plates were incubated at 35 °C for 36 h, after which *C. neoformans* colonies from two dilutions were counted and averaged.

At the end of every passage, four or five *C. neoformans* liver colonies from every passaged line were washed three times with PBS and frozen down for storage in mixture of 500 µl of 50% glycerol and 500 µl of ×2 freezing media (×2 Dulbecco's modified Eagle's medium, 20% foetal bovine serum and 10% dimethyl sulphoxide). After the final passage, 500 µl of both the liver and brain homogenate were stored separately in a 1 : 1 ratio with 50% glycerol and at −70 °C.

(e) *Tests for the evolution of virulence*

At the end of the passages, the B replicate of each post-passaged line of H99 was grown *in vitro* and used to infect five mice of each of the six strains. Mortality was extremely high (usually before day 21 post-infection) when mice were initially infected at the original dose (2.5×10^7 CFU ml^{-1}). Thus, day of death was used as a measure of virulence for these infections. In addition, four of the B-replicate post-passaged lines ($H99^B$, $H99^F$, $H99^{D/Q}$ and $H99^Q$), one of the A-replicate lines ($H99^B$) and the Balb/c passaged B replicate ($H99^{Balb}$) were used to infect mice at a lower dose (2.5×10^6 CFU ml^{-1}) to determine whether there were any MHC-specific or genotype-specific titre differences. In these infections, all mice that were still alive were killed on day 21 post-infection; livers and brains were collected and *C. neoformans* titres were determined (as in § 2d).

(f) *Statistics*

A Cox proportional-hazard regression analysis was used to test whether mortality increased during passage, whether mortality rates were different between passaged MHC genotypes and whether mortality rates were different between replicate lines of the same MHC. To confirm this analysis and to show that the replicate trajectories were more similar than expected by chance, a parametric survivorship model was used to test for significantly different trends in mortality rates during the course of passage in the different mouse genotypes. In particular, we wished to show that the patterns in individual genotypes were similar in the two replicates. We fitted the data to a parametric survivorship model (with the extreme value distribution) using either passage number or passage number and genotype as covariates, and computed the χ^2 statistic showing the improvement in fit gained by including genotype. A χ^2 value was given because the regression used in this model was distributed approximately as a χ^2 distribution. We then randomly shuffled the genotypes of the replicates and repeated the calculation. If the effect of genotype was consistent in the two replicates, the χ^2 value would be greater in the actual data than in the randomized data. A multivariate analysis of variance (MANOVA) was used to test for MHC-specific differences in titres. Because a MANOVA controls for an increase in type I error, this was followed by simple contrasts to determine which genotype(s) was responsible for the effect.

3. RESULTS

There was a significant decrease in time to death (increased mortality) during passage for all of the lines of H99 (figure 1*a*). All of the passaged lines became more virulent during the passages (Cox proportional-hazard regression analysis, $p = 0.0001$; parametric survivorship model with the extreme value distribution, $p < 0.001$) and were causing death after 7–28 days of infection, compared with 100% survival at day 39 (when they were killed) with the pre-passaged strain.

There were significant differences in mortality rate between passaged MHC lines (Cox proportional-hazard regression analysis, $p = 0.0001$; figure 1*a*), but no replicate lines differed from each other (Cox proportional-hazard regression analysis, $p = 0.95$; figure 1*b*), suggesting that MHC affects the trajectory (slope) of virulence evolution. To illustrate the magnitude of the difference between pre- and post-passaged lines, we grouped post-passaged lines into two categories: low-virulence lines (*f*/*f*, *b*/*b* and *k*/*k*) and high-virulence lines (*q*/*q*, Alt D/Q, Balb/c, *d*/*d* and Alt Het) (figure 1*c*). We were unable to evaluate liver titres during the passages since the day of death was a confounding factor in the analysis (data not shown).

There were significant differences in liver and brain titres on day 6 post-infection between the pre-passage H99 and the post-passaged $H99^{Q.B}$. H99 liver (4.16×10^4 CFU ml^{-1}) and brain (3.38×10^2 CFU ml^{-1}) titres were significantly smaller than the liver (7.24×10^6 CFU ml^{-1}) and brain (1.51×10^4 CFU ml^{-1}) titres of $H99^{Q.B}$ ($F_{1,8} = 114.3$, $p < 0.0001$), indicating that increased virulence was correlated with higher pathogen titres.

When different genotypes of MHC-congenic mice were infected with four post-passaged lines at the low dose, there were no MHC-specific titre differences for three out

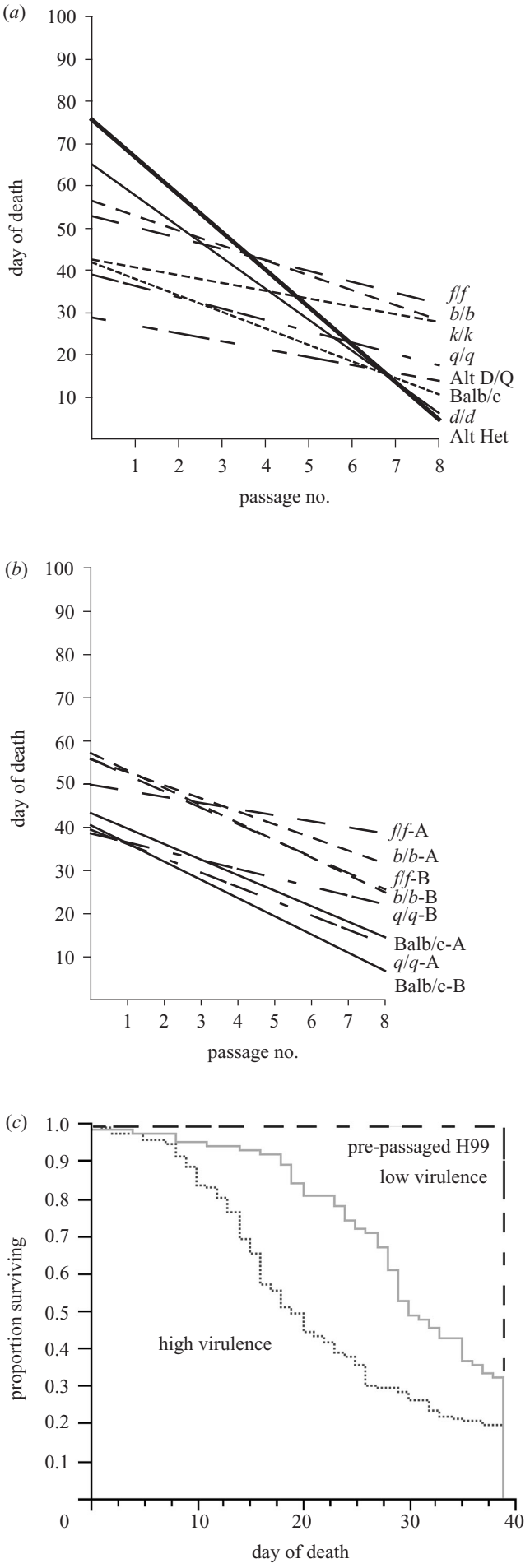

Figure 1. (*a–c*) Increase in mortality during passage. (*a*) Passage of *Cryptococcus neoformans* in different MHC-congenic strains (solid and dashed lines) produced a group of 'low'- (*f*/*f*, *b*/*b* and *k*/*k*) and 'high'- (*q*/*q*, Alt D/Q, Balb/c, *d*/*d* and Alt Het) virulence lines. (*b*) Replicate lines of passaged strains showed similar patterns in mortality during passage (replicates of *d*/*d* and *k*/*k* not shown for clarity). (*c*) MHC affects the time to death during passage with *C. neoformans*. Pre-passaged H99 is shown for comparison. Low-virulence (*f*/*f*, *b*/*b* and *k*/*k*) lines showed a longer time to death than high-virulence (*q*/*q*, Alt D/Q, Balb/c, *d*/*d* and Alt Het) lines.

of four lines tested (figure 2*a–c*). However, when infected with the A replicate of H99^{B}, *b*/*b* mice had significantly higher brain titres than the other MHC genotypes tested ($F_{5,24} = 3.96$, $p = 0.0092$; figure 2*d*). Also, when infected with the B replicate of H99^{B}, *b*/*b* mice tended to have higher lung titres than the *f*/*f* ($F_{1,24} = 2.97$, $p = 0.09$) and *q*/*q* ($F_{1,24} = 3.86$, $p = 0.06$) MHC mice (figure 2*e*). However, for the B replicate, there were no significant MHC-dependent differences in brain titres, and, unfortunately, lung titres were not quantified for the infection with the A replicate of H99^{B}. Figure 2*f* shows the liver and brain titres of MHC-congenic mice infected with H99 passaged in Balb/c mice (H99^{Balb}). There were no significant MHC-dependent differences and no significant differences between background genotypes for either liver or brain titres.

The lines of *C. neoformans* that were passaged in alternating MHC genotypes showed an increase in host mortality during passage. This was inconsistent with the retarded adaptation that was predicted to occur when the target of adaptation changed with each passage. Interestingly, both alternating passages were in the high-virulence group (figure 1*a*) and one of them, H99^{Het}, had significantly higher titres during the passages than H99^{D} ($F_{1,15} = 9.16$, $p = 0.0028$) and H99^{B} ($F_{1,15} = 9.43$, $p = 0.0024$) (data not shown).

No lines of *C. neoformans* passaged in a B10 background showed the predicted attenuation of virulence when used to infect Balb/c mice, and several showed the opposite pattern. Mortality was increased for the Balb/c when infected with H99$^{D/Q}$ (all Balb/c mice died whereas only 10% of B10 mice died: Fisher's exact test, $p = 0.0005$; figure 2*a*) and there were significantly higher liver and brain titres in Balb/c when infected with H99^{F} and H99^{Q} (figure 2*b*,*c*).

4. DISCUSSION

As expected, all of the post-passaged lines increased in virulence during serial passage as measured by mortality (figure 1*a*). MHC affected the trajectory of this virulence evolution as shown by significant differences in mortality rates between lines passaged in different MHC strains (figure 1*a*) while the independent replicate lines within MHC strains showed similar patterns of mortality (figure 1*b*).

Despite this influence of MHC on the trajectory of virulence evolution, final tests of post-passage pathogens showed no, or only small, MHC effects. Five post-passaged lines were inoculated at a low dose (2.5×10^{6} CFU ml^{-1}) to test for MHC-specific titre

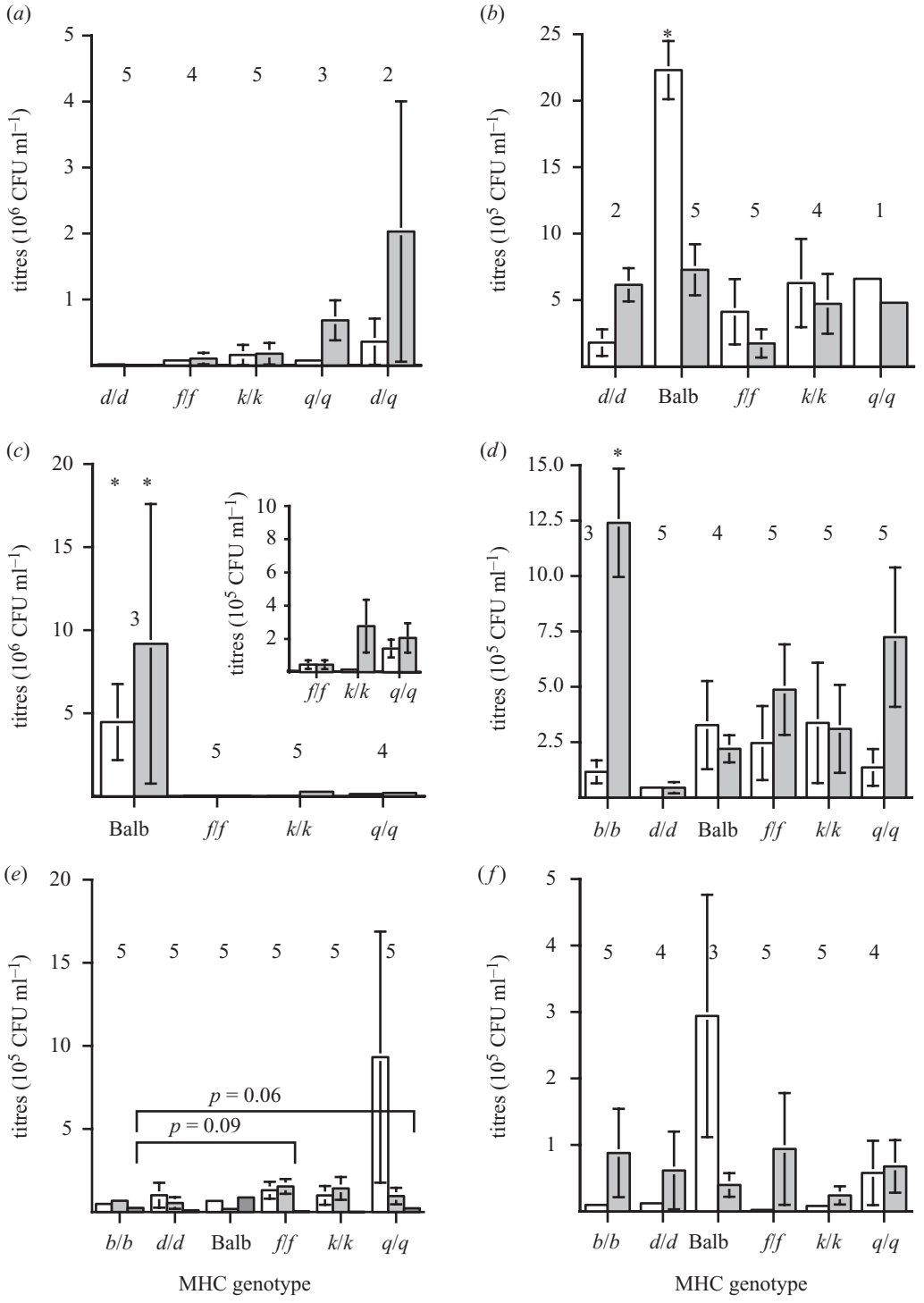

Figure 2. Liver (open bars) and brain (light-grey bars) titres of MHC-congenic mice infected with 2.5×10^6 CFU ml^{-1} of different post-passaged strains of *Cryptococcus neoformans*. Different MHC-congenic strains infected with (*a*) H99$^{D/Q}$, (*b*) H99^{F}, (*c*) H99^{Q}, (*d*) the A replicate of H99^{B}, (*e*) the B replicate of H99^{B} (lung, dark-grey bars) and (*f*) H99^{Balb}. Sample sizes are indicated above each bar. An asterisk indicates statistical significance ($p < 0.05$). Five Balb/c mice were infected with H99$^{D/Q}$ (*a*), but because they all died before day 21, there are no liver and brain titre data for these mice.

differences. Four out of the five lines tested showed no MHC-dependent differences in liver or brain titres (figure 2). In addition, when all of the post-passage strains were tested on each B10 host strain there were no MHC-specific mortality differences (data not shown). These titre and mortality results are inconsistent with the prediction that pathogens will adapt to the host genotype of passage. The one exception was H99^{B} (figure 2*d*,*e*). The fact that the only significant evidence for MHC-dependent adaptation was found in a low-virulence line suggests that MHC effects may be most easily visible in lines with low overall increases in virulence because these lines will be in the host longer and fewer non-MHC virulence factors will be present to mask the MHC effect. These results from MHC-congenic lines provide, at best, weak support for the prediction that pathogens will adapt to the host MHC genotype of passage such that virulence will be highest in that host (Ebert & Bull 2003).

One explanation for the general lack of MHC-specific adaptation as measured by pathogen titres is the possibility that our selection regime during passage favoured increased pathogenicity without increased pathogen growth rate. This possibility is rejected by our observations that post-passage strains showed higher pathogen titres when compared with pre-passage stock.

A stronger test for host-specific adaptation involved comparing passages in B10 with those in Balb/c where the host strains differ at many loci. Significant host-specific adaptation was observed in three out of six comparisons, but its direction was opposite to that predicted (figure 2). Balb/c mice had significantly higher brain and liver titres than B10 mice when infected with two post-passaged lines ($H99^{Q}$ and $H99^{F}$) and higher mortality when infected with $H99^{D/Q}$. This pattern is opposite to that observed during infection with pre-passaged stock where Balb/c was one of the most resistant strains (McClelland *et al.* 2003*b*). One explanation is that pathogens often adapt to mouse defence systems that are polymorphic because of past pathogen-mediated diversifying selection (Haldane 1949). Thus, any given mouse strain will be more or less susceptible to any given adaptation depending on the polymorphism present in that strain. For instance, it has been reported that Balb/c mice produce less inducible nitric oxide synthase in the macrophage (Yoshida *et al.* 1995) and more interleukin 4 than B10 mice (Yagi *et al.* 2002), suggesting a less efficient immune response to *C. neoformans*. If pathogens adapted to either of these systems in B10, the negative effect would probably be greater in Balb/c mice, as we observed. These unexpected patterns are inconsistent with the prediction of attenuated virulence in host genotypes that differ from the host genotype of passage.

The failure to observe disproportionately increased virulence in the host genotype of passage and attenuation in other host genotypes is contrary to common theory (Ebert 1998). Since this is the first test of pathogen adaptation with *C. neoformans*, it is unknown whether these unexpected patterns of non-attenuation observed here will commonly be seen in other host genotypes. To our knowledge, this is only the third such test in a mammalian host. The first involved a parasitic nematode in laboratory mice, where the predicted patterns were observed (Dobson & Owen 1977). The second involved passaging rodent malaria in C57Bl/6J mice and then testing pre-passage and post-passaged strains in two other mouse strains (CBA/ca and DBA/2). Interestingly, similarly to our results with Balb/c mice, Mackinnon *et al.* (2002) found that the post-passaged strain of malaria also grew faster and caused more mortality and morbidity in the other strains than in the host strain of passage (Mackinnon *et al.* 2002). If this unexpected pattern observed in malaria and *C. neoformans* continues to be found in other systems, it challenges fundamental assumptions made in most models of host–pathogen coevolution (Ebert & Hamilton 1996; Hamilton 1982; Dybdahl & Lively 1998).

The lines of *C. neoformans* that were passaged in alternating MHC genotypes or in alternating MHC heterozygotes showed no reduction in virulence increase (as measured by host mortality) either during passaging (figure 1*a*), or when used to infect different MHC-congenic strains at the original dose (data not shown). This is an unexpected result if *C. neoformans* is adapting to MHC-dependent immune responses, as adaptation to one MHC should differ from adaptation to another MHC (McMichael & Phillips 1997). Thus, alternating MHC each passage should reduce the selection for adaptation to any given MHC. This is the basis for the predicted retarded evolution rate (Ebert 1998). This failed prediction suggests that most of the virulence evolution was non-MHC related.

Why does MHC affect the trajectory of virulence evolution, but not specific adaptation? MHC is known to control the immune response in a variety of ways such as: (i) conferring susceptibility or resistance (McClelland *et al.* 2003*a*); (ii) modulating a Th_1 versus Th_2 immune response (Murray *et al.* 1994); or (iii) triggering expression of different cytokine profiles (Fischer *et al.* 1999). Through any of these mechanisms, MHC can control the population size of the pathogen and therefore the potential for natural selection to influence the trajectory of virulence evolution, without resulting in MHC-specific adaptation. However, for pathogen adaptation to contribute to maintaining MHC diversity, MHC-specific adaptation must occur (Takahata & Nei 1990). The fact that only one post-passaged line showed evidence of MHC-specific differences in titres suggests that escape of MHC-mediated immune recognition was absent or relatively weak compared with other evolving virulence factors.

The general failure to find MHC-specific adaptation seemed surprising, since every line of *C. neoformans* was passaged for between 500 and 1000 generations in different MHC backgrounds, allowing considerable opportunity for MHC-specific adaptation to occur. There are a few possible explanations. First, MHC-specific adaptation might have occurred, but overall virulence evolution was so strong that it masked the MHC effects. Small fitness differences can have significant evolutionary effects, but can be difficult to detect during experimental analysis owing to a lack of statistical power. Second, it is possible that *C. neoformans* has just adapted to 'mouse' (since it was originally isolated from a human patient) and any MHC-specific adaptation will require more passages (a longer time spent in the host). Third, it is possible that larger pathogens such as *C. neoformans* have an unlimited number of effective MHC-presented epitopes (Potts & Slev 1995). Consequently, pathogen escape of any subset of these epitopes will not lead to any long-term MHC-specific adaptation, because formerly subordinate epitopes become immunodominant (Yewdell & Bennink 1999) and the effectiveness of the immune response is largely unchanged.

Most of the increase in virulence was caused by non-MHC factors. This could be because non-MHC factors are generally more important than MHC factors, at least for this particular pathogen. Alternatively, since *Cryptococcus* is an opportunistic pathogen with a broad host range, when it is forced to adapt to a single host species, most of the initial virulence evolution may be directed towards non-MHC factors because this is where the majority of fitness advantages are. Such patterns have been found during the experimental evolution of *Chlamydomonas* to novel environments: initial adaptations differ in type from later adaptations (Kaltz & Bell 2002). It is impossible to distinguish between these possibilities

with this dataset. However, these results do suggest that serial-passage studies will be a powerful approach for identifying previously unknown virulence factors, as seen for influenza (Brown *et al.* 2001). The fact that pathogen adaptation was host-genotype specific, that is, different for B10 and Balb/c mice, also offers the opportunity to identify the host targets of pathogen adaptation using standard mapping procedures (Glazier *et al.* 2002). Understanding how virulence evolves has substantial importance for solving problems in the new area of emerging diseases (Woolhouse 2002).

This is the first systematic test, to our knowledge, of MHC-dependent pathogen adaptation in the yeast *C. neoformans*. All passaged lines increased in virulence. MHC influenced the evolution of virulence, but not in the ways required by models used to explain the evolution of MHC diversity (Takahata & Nei 1990). If these results were generally found, it would suggest that other proposed mechanisms, such as MHC-heterozygote advantage (McClelland *et al.* 2003*a*) or mating preferences (Penn & Potts 1999), may be more important than pathogen adaptation in driving MHC diversity. We do know that viruses can adapt to hosts by escaping MHC-dependent immune recognition (Pircher *et al.* 1990; Pewe *et al.* 1996; McMichael & Phillips 1997; Evans *et al.* 1999; Allen *et al.* 2000). Determining whether the dynamics of such MHC-dependent adaptation will lead to the negative frequency-dependent selection required to maintain MHC diversity (Takahata & Nei 1990) will require further experiments. Similarly, it will require additional serial-passage experiments with other pathogens to determine whether the unexpected results observed here regarding the evolution of virulence in *Cryptococcus* are general or exceptional.

The authors thank Jodi Harper, Maureen Wilkinson and Wes Perrine for help with mouse husbandry and mouse experiments; Jane Libbey for technical assistance; Robert Fujinami for supplies; Andy Alspaugh for media recipes; and Stuart Levitz, Arturo Casadevall, John Perfect, Joe Heitman, Gary Cox and Andy Alspaugh for help with *Cryptococcus* pathology and for constructive comments. This work was supported by a NIH grant to W.K.P. (GM39578) and partly by a Technology Innovation grant from the University of Utah to D.L.G.

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