Immunity Promotes Virulence Evolution in a Malaria Model

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Evolutionary models predict that host immunity will shape the evolution of parasite virulence. While some assumptions of these models have been tested, the actual evolutionary outcome of immune selection on virulence has not. Using the mouse malaria model, *Plasmodium chabaudi*, we experimentally tested whether immune pressure promotes the evolution of more virulent pathogens by evolving parasite lines in immunized and nonimmunized (“naïve”) mice using serial passage. We found that parasite lines evolved in immunized mice became more virulent to both naïve and immune mice than lines evolved in naïve mice. When these evolved lines were transmitted through mosquitoes, there was a general reduction in virulence across all lines. However, the immune-selected lines remained more virulent to naïve mice than the naïve-selected lines, though not to immunized mice. Thus, immune selection accelerated the rate of virulence evolution, rendering parasites more dangerous to naïve hosts. These results argue for further consideration of the evolutionary consequences for pathogen virulence of vaccination.

Introduction

Genetic variation in pathogen virulence (harm to the host) has been found whenever it has been looked for. A considerable body of theory, based on the transmission consequences of virulence, has been developed to predict how natural selection will act on this genetic variation and how it will shape virulence levels in natural populations of disease-causing organisms (Frank 1996; Diekmann et al. 2002). For instance, natural or vaccine-acquired host immunity protects hosts from dying, thereby relieving the parasite of the potential fitness costs of prematurely shortened infections. Thus, host populations with high levels of immunity can maintain more virulent pathogens than can naïve host populations (Gandon et al. 2001). To date, the best example of virulence evolving upwards in response to enhanced levels of host defense comes from an uncontrolled “experiment” in the field: upon release into a highly susceptible host population, the myxomatosis virus evolved lower virulence (Fenner and Ratcliffe 1965) but then later increased in virulence once the host population had evolved resistance (Best and Kerr 2000).

As well as altering between-host selection pressures on virulence, host immunity can alter the nature of inhost selection. Different directions of virulence evolution are expected depending on the details of inhost competition among parasites (e.g., Nowak and May 1994; Van Baalen and Sabelis 1995; Chao et al. 2000; Brown et al. 2002). Unfortunately, these details are not well understood for any pathogen (Read and Taylor 2001). The only generality is that serial passage of pathogens almost always increases virulence (Ebert 1998), implying that virulent variants have a fitness advantage within hosts. However, all serial passage experiments of which we are aware were conducted in immunologically naïve hosts, so the effects of immunity on virulence evolution are unknown. In theory, immunity could impose selection in several ways. For instance, lower parasite loads should reduce resource competition (e.g., for red blood cells) among parasites occupying the same host, but increase the competition for enemy-free space (e.g., by immune evasion). This could lead to more aggressive parasites racing to stay ahead of proliferating immune responses (Antia et al. 1994); it could also lead to the evolution of novel antigenic variants that have a selective advantage only in immunized hosts. Immunization will also alter the timing of immune selection, thus potentially selecting for changes in parasite life history parameters that affect virulence, such as an earlier or higher rate of production of transmission stages (Koella and Antia 1995). Finally, the rate at which virulence evolution occurs may be limited by the size of the parasite population inside the host, and therefore may be retarded by host immunity. Thus, at least in theory, there are many potential consequences for virulence evolution of prior host immunity, both long-term and short-term in nature.

One barrier to testing theoretical models of virulence evolution is that the models typically predict the outcome at evolutionary and epidemiological equilibrium. New equilibria may or may not take a long time to reach, but will in any case depend on the dynamics of the host population and the environmental conditions under which transmission occurs: this means that experimental evolution to new equilibria will be hard to study in the laboratory for medically relevant pathogens. However, the short-term consequences for virulence evolution, which are at least as important to public health policy as the long-term consequences, may be more tractable. This is especially true for diseases for which animal models are available.

In this study, we begin the empirical effort to determine the likely direction of immune-mediated virulence evolution...
by performing experimental evolution of the rodent malaria parasite, *Plasmodium chabaudi*, in laboratory mice. We evolved multiple lines of *P. chabaudi* in immunized and naïve mice by repeated serial passage of blood-stage parasites (i.e., bypassing the normally obligate mosquito vector) starting from two different starting populations. After 20 passages, the lines had evolved sufficiently to make comparisons between the immune-selected lines (I-lines) and naïve-selected lines (N-lines) for virulence and transmissibility.

**Results/Discussion**

We found that both the I-lines and N-lines evolved to become more virulent than their ancestral populations, but the I-lines became even more virulent than the N-lines (Figure 1A). This higher virulence was manifest in both naïve and immunized mice. When the lines were transmitted through mosquitoes, there was generally a reduction in virulence across all the lines, but the I-lines remained more virulent than the N-lines to naïve mice, though not to immunized mice (Figure 1B). We discuss these two principal findings separately below.

**Immunity Selects for Higher Virulence**

The results suggest that immune selection on blood-stage parasites is more efficient at selecting virulent variants than is selection in naïve mice. Response to selection is a function of the amount of variation in the population and the proportion of the population that survives to produce offspring, i.e., the selection intensity. The higher selection response in the I-lines is unlikely to be due to greater variation on which selection could act because the parasite population size on the day of transfer in immunized mice was on average 2-fold smaller than in naïve mice (Figure 2). It is also unlikely to be due to lower host death in the I-lines as there were no line differences in mortality in naïve mice over the entire course of the experiment (10/223 naïve mice infected with N-lines versus 2/40 naïve mice infected with I-lines, *p* > 0.10 by 2-tailed Fisher’s Exact test, zero mortality in immunized mice), and all but one of the deaths occurred after the day of transfer. The most likely explanation is that immunity generated more intense selection by killing a greater proportion of the parasite population up until the point of transfer (Figure 2). Winners of the race into the syringe on day 7 were those parasite variants that survived immune selection, and these parasites proceeded to cause more damage to their host later in the infection.
But why would selection favor more virulent parasites? Our previous studies have consistently shown that peak parasite densities in the acute phase are positively correlated to the level of virulence that they generate (Mackinnon and Read 1999a, 1999b, 2003; Mackinnon et al. 2002; Ferguson et al. 2004). We therefore expected to find that the higher virulence in I-lines was accompanied by higher parasite densities, in which case we would deduce that immune selection had favored variants that were better able to outgrow immune defenses. While we found positive relationships between asexual multiplication and virulence across all the lines including the ancestral ones (Figure 3A), the I-lines and N-lines were statistically indistinguishable ($p > 0.05$) for (i) parasitemia on day 4, (ii) parasitemia on day 6 or 7, (iii) the increase in parasitemia from day 4 to day 6 or 7, and (iv) maximum parasitemia, with one exception: maximum parasitemia was significantly higher in I-lines than N-lines derived from unadapted ancestors when measured in immunized mice, and this only in one of the two replicate experiments (23% versus 6.9% parasitemia, $p < 0.001$). Thus, there is little evidence to suggest that the increased virulence was due to a higher asexual multiplication rate (or a lower death rate of asexuals) in those parasites that successfully made it into the syringe. Our data demonstrate that immunity acts as a powerful and upward inhost selective force on virulence, but the precise mechanism awaits further study.

There were positive relationships between virulence and lifetime transmission potential across all the lines (Figure 3B), consistent with our previous studies (reviewed in Mackinnon and Read 2004), but the differences between the I-lines and N-lines were not statistically significant ($p > 0.05$). Gametocyte densities are a good predictor of transmission probability in *P. chabaudi* and other *Plasmodium* species (Mackinnon and Read 2004), so these results demonstrate that the more virulent parasites evolved in semi-immune mice would transmit as successfully as the less virulent parasites evolved in naive hosts. Thus, in the absence of a cost, virulent variants favored by within-host immune selection are expected to spread throughout an immunized host population.

**The Effects of Mosquito Transmission**

Malaria parasites, like many microbes (Ebert 1998), are remarkable in their ability to rapidly adapt to changes in their host environment, and some of this is known to be due to phenotypic switching mechanisms in virulence-related phenotypes such as binding to host cells (Barnwell et al. 1983), red cell surface antigen expression (Brown and Brown 1965;
mission are due to the systematic resetting during meiosis and virulence reductions observed following mosquito transmission and infectivity of sporozoites in the mosquito (Ebert 1998): the potential against virulent variants that have lost or reduced the ability to transmit through mosquitoes (Ebert 1998); the potential against virulent variants that have lost or reduced the ability to transmit through mosquitoes (Ebert 1998). Alternatively, virulence reductions following mosquito transmission favors this hypothesis. Possible reasons for this are discussed further below. For now, we note that the data are consistent with (though do not directly test) the prediction (Gandon et al. 2001) that enhancement of host immunity by anti-blood-stage vaccination will render malaria populations more dangerous to naive hosts, at least in the short- to medium-term. Whether or not our long-term prediction (Gandon et al. 2001) that immunized populations will drive virulence to a higher level at evolutionary equilibrium proves true can be established only by monitoring vaccine-covered parasite populations in the field.

We observed a general reduction in virulence across all lines following mosquito transmission (see Figure 1), particularly when measured in immunized mice, and particularly in lines that had been selected under immune pressure, i.e., the I-lines, and in the CW-A ancestral line, which had been serially passaged on day 12 postinfection (PI). Many laboratory studies in malaria have shown that high or low virulence phenotypes accrued through serial passage can be maintained upon transmission through mosquitoes (James et al. 1936; Coatney et al. 1961; Alger et al. 1971; Walliker et al. 1976; Knowles and Walliker 1980; Walliker 1981; Barnwell et al. 1983), although occasional major losses (or gains) of virulence do occur (Alger et al. 1971; Walliker et al. 1976; Knowles and Walliker 1980; Gilks et al. 1990). Mosquito transmission could play a significant role in virulence evolution that is driven by inhost selective processes (as distinct from the between-host selective processes underlying the vaccination hypothesis in Gandon et al. [2001]).

The mechanistic basis for the reduction in virulence following mosquito transmission remains to be determined. We offer the following speculations. It may be that the virulence reductions we and others have observed are due to stochastic loss of virulent variants during the population bottlenecks that occur during mosquito transmission (the variability between lines in virulence loss during mosquito transmission favors this hypothesis). Alternatively, virulence reduction may be due to the deterministic forces of selection against virulent variants that have lost or reduced the ability to transmit through mosquitoes (Ebert 1998): the potential trade-off between virulence in the vertebrate host and production and infectivity of sporozoites in the mosquito has not yet been explored. A further possibility is that the virulence reductions observed following mosquito transmission are due to the systematic resetting during meiosis of the expression of genes that have been switched on or up-regulated during asexual serial passage.

To what extent do our observations accord with previous work on serial passage of malaria in immune-modified environments? Results from other studies are difficult to interpret as none maintained control lines for selection (i.e., lines that were passaged in the nonmanipulated immune environment), most had no replication of lines within selection treatment, and some used just a single selection step. Nevertheless, some tentative conclusions may be drawn. Comparisons of selected and ancestral parasites have been made after three different forms of immune manipulation: (i) down-regulation of immunity by removal of the spleen prior to infection, (ii) up-regulation of immunity by transfer of immune serum at the beginning of infection, and (iii) up-regulation of immunity by infection, sometimes with sub-curative drug treatment in order to establish a chronic infection. In the first two, parasites were selected from the primary wave of parasitemia, as in our experiment, whereas in the third, selected parasites were isolated from relapses much later in the infection (40–150 d PI). Parasite lines passaged through splenectomized hosts often lose the ability to bind to host endothelial cells (cytoadherence) in the microvasculature of the deep tissues and therefore the ability to avoid being passed through the spleen (Garnham 1970), the primary site of immune-mediated clearance (Wyler 1983). This loss of binding is often accompanied by a loss of ability
to express (Barnwell et al. 1983; Handunnetti et al. 1987; Gilks et al. 1990)—or a major alteration in the level of expression of (David et al. 1983; Fandeur et al. 1995)—the highly variable and clonally variant switching parasite antigens on the surface of the red cell known to be important for the maintenance of long-term chronic infections (Brown and Brown 1965). In P. falciparum at least (David et al. 1983; Hommel et al. 1983), this coincident change in the two properties is because both phenotypes are mediated by the same parasite molecule, denoted PiEMP1 (Baruch et al. 1995; Smith et al. 1995; Su et al. 1995). Importantly, in two of three studies, the line of parasites that lost cytoadherence and/or surface antigen expression had much-reduced virulence to spleen-intact naïve hosts compared to their ancestral lines (Barnwell et al. 1983; Langreth and Peterson 1985; Gilks et al. 1990). If our immunization procedure was priming the spleen for effective parasite clearance, our results are consistent with these findings.

However, the second form of immune selection—passage of acute-phase parasites from hosts injected with antiseraum at the beginning of the infection—yielded parasites with lower virulence to naïve mice than their ancestors in one study (Welldle and Diggs 1978), although it had no impact on virulence in two other studies (see Briggs and Welldle 1969). The third type of immune selection—selection of virulence from relapses late in the infection—had generated parasites with virulence to naïve hosts that is lower than Cox (1962), higher than (Sergent and Poncet 1953), or similar to (Cox 1959) that of their ancestors. In all these studies, which involved only single passages, selected parasites were more virulent than their ancestors to immunized hosts, suggesting that the selected parasites were predominantly of a novel antigenic type (a fact that has sometimes been demonstrated; Voller and Rossan 1969). Whether antigenic novelty is traded off against multiplication rate or virulence among the repertoire of variants expressed during a single infection—as has also been suggested from field population studies (Bull et al. 1999)—is an interesting question that deserves more attention. However, in our study, in which we focused on the longer-term and more natural environment of hosts pre-immunized with a heterogeneous parasite population, the higher virulence of the I-lines compared to the N-lines in both naïve and immunized mice leads us to deduce that selection associated with virulence overrides selection for immune evasion alone.

Conclusion

Our data demonstrate that host immunity can increase the potency of inhost selection for higher virulence in malaria. Whether our results generalize to other immunization protocols, parasite clones, parasite species, host genotypes, repeated mosquito passage, and so on requires extensive further experimentation. But, coupled with the malaria parasite’s famous ability to rapidly adapt to novel conditions in the laboratory (see above) and to variant-specific vaccine pressure (Genton et al. 2002) and drugs (Peters 1987) in the field, these results urge the continuous monitoring of virulence of parasite populations if asexual-stage malaria vaccines become widely used. And for other microparasites (bacteria, viruses, and protozoa) that rely on rapid multiplication within the host for successful transmission, similar concerns might apply.

Materials and Methods

Selection phase. Starting from two separate ancestral lines derived from clone CW (see below), five parasite lines (“sublines”) from each ancestral line were repeatedly passaged in mice (female C3HBl/lC-7—10 mice that were naïve to malaria infection) five from each ancestral line were passaged in immunized mice (I-lines, see below), forming 20 lines (“sublines”) in total. Passages involved the syringe transfer to a fresh mouse of 0.1 ml of diluted blood containing 3 × 10⁵ parasites from a donor mouse that had been infected 7 d previously. Day 7 PI is during the period of rapid population growth, and is about 2 d prior to peak parasitemia, after which population size rapidly declines (see Figure 2). Parasite lines under the same selection regime (i.e., passage in immune versus naïve mice) were not mixed at each transfer, thus yielding five independent replicate sublines in each of the four selection treatment—ancestral line groups.

Immunization was by infection with 10⁵ parasites of a different clone (denoted ER), followed by drug cure with 10 mg/kg of mefloquine for 4 d starting on day 5 PI. Naïve mice were injected with parasite-free media but were not drug treated. Re-infection took place on average 3 wk after the end of drug treatment (range 1.5–5 wk): as the half-life of mefloquine in mice is reported to be 18 h (Peters 1987), the residual amount in the blood by this stage was expected to be very low. The same deep-frozen stock of ER was used for all experiments. ER is genetically distinct from CW at marker loci (data not shown) and was originally isolated from different hosts. Before use in this experiment, ER had undergone two passages since mosquito transmission and more than 20 passages prior to that. No recrudescence infections in immunized mice were detected prior to challenge. In generations 10 and 11, all lines were passaged through naïve mice.

The serial passage experiments in this study were replicated using two different starting populations (ancestral lines)—one avirulent (CW-0) and one virulent (CW-A). CW-0 had been cloned by serial dilution from an isolate obtained from its natural host—the thicket rat, Pseudomys auratus, and then blood passed every 12 d to a total of 12 passages to produce the CW-A line. During these passages, CW-A was subjected to selection for low virulence on the basis of how much weight loss it caused to mice. Despite this selection, however, CW-A increased in virulence relative to CW-0 during the passages (Mackinnon and Read 1999b). Prior to use in the current experiments, both CW-0 and CW-A underwent four further serial passages in naïve mice, and were not recloned.

All the lines, including the ancestral lines, were transmitted once through Anopheles stephensi mosquitoes by allowing 50–100 mosquitoes aged 2–5 d to take a blood meal for 20–30 min on an anaesthetized gametocytic mouse that had been inoculated 6–10 d previously, i.e., prior to the peak of infection. Then, 11–12 d later these mosquitoes—typically 10–20 of them infected as assessed by random plating of oocysts—were allowed to feed back onto anaesthetized naïve mice. After 7–10 d, the blood from these sporozoite-infected mice was harvested and stored in liquid nitrogen. These aliquots were used to initiate blood infections in naïve mice that were then used as donors of asexual parasites to mice involved in the posttransmission experiments. As the lines were transmitted through mosquitoes noncontemporaneously, and involved typically one mouse per subline, comparisons among the lines for infectivity to mosquitoes were not made during these transmission exercises.

Evaluation phase. After 18 passages, the pretransmission lines were evaluated in two replicate experimental blocks in naïve (generations 19 and 21) and immunized mice (generations 20 and 22). Ancestral lines were only evaluated in generations 21 and 22. This set of trials was denoted the “pretransmission experiments.” In a separate set of experiments, the “posttransmission experiments,” the mosquito-transmitted lines were compared with each other, as well as with the nontransmitted ancestral lines in two replicate experimental blocks in both naïve (generations 23 and 24) and immunized mice (generations 25 and 26). In both these experiments, across both blocks, ten mice were used for each of the four selection groups (two per subline), and five mice were used per ancestral line. Red blood cell density was measured every 1 or 2 d for the first 18 PI by flow cytometry (Coulter Electronics, Luton, United Kingdom), and the minimum density reached was taken as a measure of virulence. Liverweight of the mouse was also recorded every 1–2 d. During the pretransmission experiments (generations 19–22), parasitemia and gametocytemia (proportions of red blood cells infected with asexual parasites and gametocytes, respectively) were evaluated from Giemsa-stained thin blood smears every 2 d from day 4 PI until day 18 PI, and then four more times until day 43 PI. Total lifetime transmission
potential was measured as the average gametocytemia throughout the infection from day 4 to day 18 PI.

**Analysis.** Statistical analyses were performed separately for the pretransmission and posttransmission experiments as these were carried out at different times. The virulence measure used for the final analysis was minimum red blood cell density, though other measures of virulence were also analyzed (unpublished data). Since selection treatment was replicated on sublines, thus making subline the independent experimental unit, the means of mice within sublines were first calculated. These were then analyzed for the effects of immune environment on selection response by fitting a linear model to these data with factors for selection line (with three levels for nontransmitted ancestral lines, N-lines, and I-lines in the case of the pretransmission experiments), and four levels for the transmitted versions of these three lines plus the nontransmitted ancestral population (CW-0, CW-A), and an interaction between these two factors. Thus, statistical tests of differences between the selection lines and other factors in the model were made using t-tests, with the variance for subline means as the residual. An alternative model fitted to data on individual mice (rather than means of sublines) that incorporated subline as a random effect was found to be unsatisfactory because in some treatment groups, the model did not converge and estimates of the subline variance were highly variable between groups. To determine the effects of mosquito transmission on the differences in virulence, a further analysis was performed on the combined data from the pretransmission and posttransmission experiments fitting a fixed effect factor of line-within-experiment in the statistical model (seven levels—three lines for the pretransmission experiment and four for the posttransmission experiment). These analyses were carried out separately for each of the four immune-treatment-by-ancestral-line groups. Since the pretransmission ancestral line was included in both the pretransmission and posttransmission experiments, the effect of mosquito transmission (and its standard error) on the N-lines and I-lines, which was not measured directly (i.e., in a single experiment), could be estimated by reference to this line. For example, the effect of mosquito transmission in the N-lines was estimated from the difference between the N-lines and their pretransmission ancestral line in the pretransmission experiment minus the analogous contrast in the posttransmission experiment. This was done using the method of linear contrasts provided for in the SAS GLM procedure (SAS 1990). The effect of mosquito transmission on the difference between the I-lines and N-lines was similarly calculated but without reference to the pretransmission ancestral line. The effect of mosquito transmission on the ancestral lines was estimated from the direct comparison available from only the posttransmission experiment data.

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