PART FOUR

GENETIC AND EVOLUTIONARY CONSIDERATIONS

ELEVEN

The Evolution of Pathogen Virulence in Response to Animal and Public Health Interventions

Andrew F. Read, Sylvain Gandon, Sean Nee, and Margaret J. Mackinnon

INTRODUCTION

Pathogen evolution poses the critical challenge for infectious disease management in the twenty-first century. As is already painfully obvious in many parts of the world, the spread of drug-resistant and vaccine-escape (epitope) mutants can impair and even debilitate public and animal health programs. But there may also be another way in which medical and veterinary interventions can prompt pathogen evolution that erodes the effectiveness of those interventions. Virulence- and transmission-related traits are intimately linked to pathogen fitness and are almost always genetically variable in pathogen populations. They can therefore evolve. Moreover, virulence and infectiousness are the target of medical and veterinary interventions. Here, we focus on vaccination and ask whether large-scale immunization programs might impose selection that results in the evolution of more-virulent pathogens.

The word virulence is used in a variety of ways in different disciplines. We take a parasite-centric view as follows. We use "disease severity" (morbidity and/or mortality) to mean the harm to the host following infection. Disease severity is thus a phenotype measured at the whole-organism (host) level that is determined by host genes, parasite genes, environmental effects, and the interaction between those factors. One component of this is virulence, a phenotypic trait of the pathogen whose expression depends on the host. Thus, virulence is the component of disease severity that is due to pathogen genes, and it can be measured only on a given host. We assume no specificity in the interaction between host and pathogen (more-virulent strains are always more virulent, whatever host they infect).

In "Malaria Virulence," we return to that assumption, and the evidence that there are more-or less-virulent pathogens within species.

We begin by asking how natural selection might shape virulence, and in light of that, we ask how widespread immunization might alter selection on virulence. We are deeply skeptical that there will be any simple generalizations about virulence evolution that will apply across all infectious diseases, and so we ground our argument in the biology of malaria, with which we are most familiar. Nonetheless, widespread vaccination has been used for a range of human and animal diseases for much of the twentieth century and our arguments may apply to some of these. In the second half of this chapter, we therefore consider infectious diseases other than malaria.

NATURAL SELECTION ON VIRULENCE

A large number of both host and pathogen genes are responsible for disease severity, and in almost all cases these genes will be interacting with each other and a very large number of environmental determinants such as host age, condition, and previous exposure. Understanding the nature of selection on genetic determinants of disease severity is clearly a mammoth task. But it is certainly possible to understand the evolution of traits under environmental and polygenic control (for example, body size, life span, and resistance), so we see no reason in principle why it should not be possible to make progress analyzing components of disease severity, such as virulence. In the future, it should become possible to analyze the natural selection on particular phenotypes contributing to overall virulence, such as pathogen replication rates and immune evasion. The extent to which analysis of individual components will be useful will depend on their contribution to variation in virulence and in turn to disease severity.

There is now a large body of theoretical work examining the evolution of virulence. The classical framework we use is the best developed theoretically, but most importantly it has the great merit that its assumptions and predictions are empirically testable. Nevertheless, we recognize that a challenge for the future is to incorporate such complexities as host genetic diversity and coevolution. The idea is that excessively virulent pathogen mutants are eliminated by natural selection because they kill their hosts and therefore themselves. Excessively avirulent mutants also have low fitness because they are more rapidly cleared from their hosts or they fail to maximize their output of transmission propagules. Natural selection should therefore optimize the balance between the costs of virulence (host death and/or morbidity) and the benefits (immune evasion and host resource extraction) (May and Anderson 1983; Bull 1994; Ewald

1994; Frank 1996; Ebert 1999; Read et al. 1999; Day 2001; Dieckmann et al. 2002). Empirical support for this trade-off framework comes from a number of animal and plant models (Bull et al. 1991; Bull and Molineux 1992; Herre 1993; Ebert and Mangin 1997; Fenner and Fantini 1999; Messenger et al. 1999). Its usefulness in a medical context has recently been questioned (Ebert and Bull 2003), but it seems to us that its utility in any disease context will depend on the extent to which the disease in question fulfills the assumptions involved in the framework. In particular, are there virulent and avirulent parasites in a population? Second, are the fitness costs and benefits of virulence as assumed by the trade-off model? These are disease-specific issues.

MALARIA VIRULENCE

Parasite Genetics?

Malaria disease severity is determined by a complex interaction of host and parasite genetics as well as factors such as previous exposure and socioeconomics (Mbogo et al. 1999; Mackinnon et al. 2000; Phillips 2001; Greenwood and Mutabingwa 2002; Miller et al. 2002). Malaria has of course provided some of the best examples of host genes involved in disease severity, and there is currently great enthusiasm about exploiting information from the human genome project to identify further such genes. However, it is very striking that despite the vast sums of money involved, there has been little attempt to use the tools of quantitative genetics to determine what proportion of the variation in disease outcome is actually due to host factors. In the only such study we know of, a pedigree analysis of malarial disease severity in human populations in Sri Lanka, as little as 10% of the variance was due to host additive genetic factors, and perhaps as much as another 10% was due to nonadditive genetic factors (Mackinnon et al. 2000). Environmental factors such as previous disease episodes, distance from water bodies, and housing type accounted for up to another 20%. The remaining unexplained variance could be due to unmeasured environmental effects or noise. It could also be due to parasite genetic variation. It is true that polymorphisms in many of the human genes most associated with malaria disease are rare or do not exist in Sri Lanka, so an analysis elsewhere may demonstrate a greater role for host genetics. Nonetheless, this early study does make clear that the evolutionary processes determining malarial disease severity need not involve only the human genome.

Plasmodium would be an extraordinarily unusual pathogen if it was not variably virulent. So far as we are aware, wherever virulence variation has

been looked for in other pathogen species, it has been found (e.g., Sibley and Boothroyd 1992; Lipsitch and Moxon 1997; Ebert 1998; Fenner and Fantini 1999; Pandey and Igarashi 2000; Ochman and Moran 2001; Read and Taylor 2001). Certainly there are substantial differences in virulence between *Plasmodium* species. However, direct evidence of virulence variation within species is actually rather limited. This is probably because the obligate sexuality of *Plasmodium* generates high rates of recombination in all but very low transmission situations, so that virulent and avirulent strains are impossible to recognize in the field. Nonetheless, some candidate virulence determinants in human *Plasmodium* species have been proposed (Marsh and Snow 1997; Hayward et al. 1999; Preiser et al. 1999; Miller et al. 2002), and over representation of some parasite alleles among severe malaria cases has been reported (Engelbrecht et al. 1995; Robert et al. 1996; Kun et al. 1998; Ariey et al. 2001). Experimentally demonstrating the involvement of particular genes has proved difficult, not least because virulence cannot be assayed *in vitro*. Nonetheless, a variety of indirect data point to genetic variation in *Plasmodium* virulence. First, deliberate infections of people demonstrated strain differences in virulence (James et al. 1936; Covell and Nicol 1951), and there were strain differences both within and between geographic regions in the speed with which infections became life-threatening (Figure 11.1) and in putative virulence determinants such as growth rates (Gravenor et al. 1995). Second, various phenotypes believed to be encoded by parasite genes are correlated with disease severity (Marsh 1992; Miller et al. 2002), such as *in vitro* proliferation rates (Figure 11.2, Chotivanich et al. 2000), rosetting (Carlson et al. 1990; Rowe et al. 1997), and cell selectivity (Simpson et al. 1999). Crucially, genetic variation for virulence has been readily uncovered in animal models of malaria (Yoeli et al. 1975; Cox 1988; Mackinnon and Read 1999a). For instance, in our laboratory, genetically distinct clones of *Plasmodium chabaudi* differ in their virulence in mice, measured as anemia, weight loss, and mortality (Mackinnon and Read 1999a; Ferguson et al., submitted). These clone differences are consistent across a range of conditions such as dose (Timms et al. 2001), host sex (Mackinnon, Personal Communication, 2002), host immune status (Buckling and Read 2001; Mackinnon and Read 2003), drug treatment (Buckling et al. 1997), mosquito passage (Mackinnon Personal Communication, 2002), the presence of competing clones (Taylor et al. 1998; Timms 2001), and host genotype (Mackinnon et al. 2002).

Taken together, then, it seems extremely likely that there is genetic variation for virulence in human *Plasmodium* populations. With the near

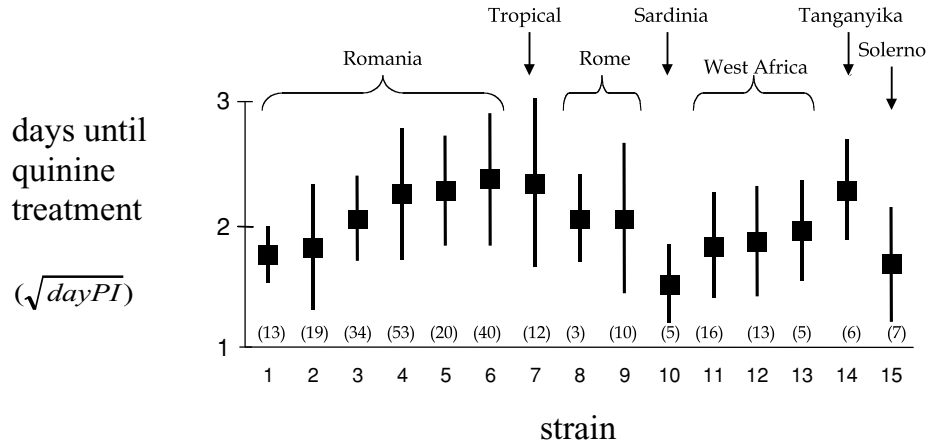

Figure 11.1. Days until quinine therapy became necessary for malaria-naive patients deliberately infected with different strains of *P. falciparum* as therapy for neurosyphilis in the Horton Hospital, UK, during the 1930s and 1940s. Quinine was administered when attending physicians considered the patient's life to be at risk. Data are available for a total of 250 patients infected with one of 19 strains; plotted points are for the 15 strains for which three or more patients were infected. The strains came from seven geographic regions, as labeled above. Sample sizes are given in brackets; plotted points are means ± 1 s.e.m. The vertical axis is the square root of days post-infection (PI). Strains from various geographic regions differed significantly in how fast they induced life-threatening disease ($F_{6,\,241} = 2.31$, $p < 0.035$), and there were also significant differences between strains within geographic regions ($F_{12,\,241} = 3.32$, $p < 0.0001$). Data sources are described by Gravenor et al. (1995); data are from M. Gravenor (personal communication, 2002).

completion of the malaria genome project, and advances in bioinformatics, it is now possible to look for genetic differences in malaria parasites from patients with severe or mild disease, and there is every reason to think that particular parasite genes will be identified in the near future. But, from our perspective, a key issue is the fitness consequences of virulence for malaria parasites.

Selection for Virulence?

It is going to be extraordinarily difficult to obtain (ethically) data on the fitness costs and benefits of virulence for a human disease. However, it has proved relatively easy to identify fitness benefits of virulence in our rodent model for malaria, *P. chabaudi*, which shares many of the life-history characteristics of the most virulent of the human species, *P. falciparum*. In the absence of host death, more-virulent clones are cleared less rapidly,

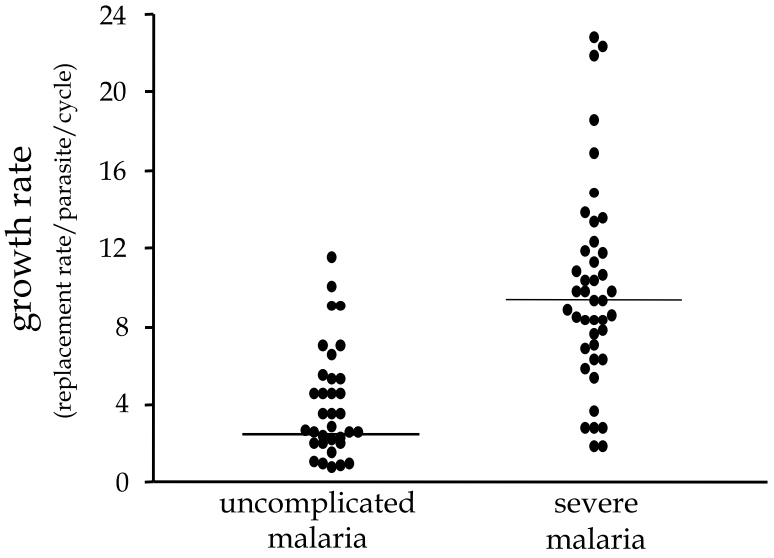

Figure 11.2. *In vitro* replication rates of *P. falciparum* parasites isolated from patients in hospitals in Thailand with life-threatening or mild malaria. Each dot represents parasites from a single patient. Redrawn from Chotivanich et al. (2000).

produce more gametocytes (transmission stages), and transmit better to mosquitoes (Mackinnon and Read 1999a, 2003; Ferguson et al., Personal Communication, 2002). Importantly for our argument below, the same advantages to virulence accrue in semi-immune hosts (Figure 11.3). Moreover, as others have found in malaria and many other systems (Ebert 1998), serial passage by syringe leads to increasing virulence (Mackinnon and Read 1999b). This implies a within-host fitness advantage: presumably, virulent variants arise that have an advantage in the race for the syringe. Thus, we have evidence of within- and between-host (transmission) advantages to virulence.

Selection Against Virulence?

If virulence only enhanced fitness, ever more-virulent malaria parasites would be favored by natural selection. There are a number of possible fitness costs to virulence. Conventional wisdom is that excessively virulent pathogens kill their hosts and therefore themselves. However, it is remarkably difficult to get quantitative data on the fitness costs of pathogen-induced host death in any system. Clearly, if death is extremely rapid, and occurs before transmission propagules are produced, then pathogen fitness will be zero. But diseases are rarely that lethal. In our mouse malaria

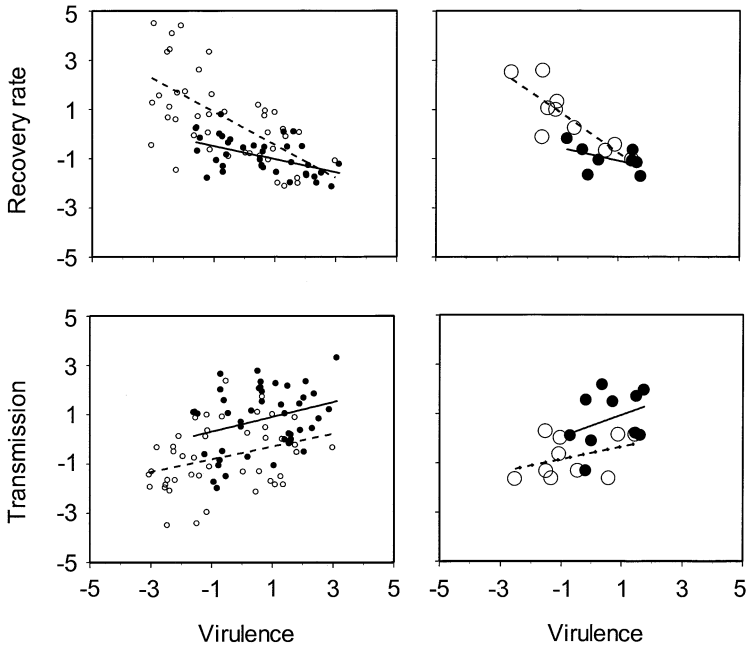

Figure 11.3. In the absence of host death, *P. chabaudi* virulence is associated with less-rapid clearance and enhanced transmission. Graphs show the phenotypic (cross-mouse, left) and genetic (cross-clone, right) relationships across immunologically naive (solid symbols and lines) and semi-immunized (open symbols, broken lines) mice. Numerical values are first principal components of traits associated with virulence (anemia and weight loss), transmission (gametocytemia), and recovery (duration of infection, rate of clearance of parasites). Mice were immunized with live parasites and cleared by chemotherapy four days later. Reproduced with permission from Mackinnon and Read (2003).

model, risk of death is at its greatest if the mice fail to control the initial proliferation of asexual parasites. Most transmission stages appear after this, so that in one of our experiments, host death resulted in a 75% reduction in transmission potential (Mackinnon et al. 2002). In humans, recent analysis of malaria therapy data suggests that failure to control initial proliferation is also a major determinant of risk of severe disease and death for *P. falciparum* (Molineaux et al. 2001), and here too, the bulk of gametocyte production occurs after the first wave of parasitemia (James et al. 1932, 1936). Thus, it seems likely that host death will impact pathogen fitness in the field. Whether this is sufficient to offset the fitness gains of virulence is very unclear. In a selection experiment with *P. chabaudi*, in which we mimicked a mortality cost of virulence by preventing parasites from 50–75% of the most virulent infections from proceeding to the

next host, virulence increased nonetheless (Mackinnon and Read 1999b). Thus, this intensity of host-level selection was insufficient to prevent the evolution of more-virulent parasites. Case fatality rates in the section of human populations responsible for the bulk of malaria transmission are hard to estimate but they may be somewhere between 10% and 1% or even less (Snow et al. 1999; Trape et al. 2002). However, the key issue is not mortality risk *per se* but rather how an increased risk of host death is traded-off against the increased transmission benefits of virulence in the absence of host death. To determine fitness functions for a human pathogen is a formidable challenge.

There are other possible costs of virulence (Ebert 1998; Day 2001). For instance, enhanced transmission to mosquitoes could be associated with reduced transmission to the next vertebrate host if strains more infectious to mosquitoes are also more lethal to them because of their greater parasite burdens. Malaria parasites can kill mosquitoes (Ferguson and Read 2002a,b), but we found no evidence that more-virulent infections in mice were more lethal to the mosquitoes they infected (Ferguson et al., Personal Communication, 2002). Another cost of virulence may be an inability to successfully infect a range of host genotypes. Pathogens that achieve high virulence on one host genotype might, for instance, achieve lower virulence on another. The existence of such a pattern is at the heart of a broad array of coevolutionary theory (Hamilton 1980; Lively and Apanius 1995), but despite some evidence of it in plants and invertebrates (Little 2002), we know of no examples from pathogens of vertebrates where strains virulent on one host genotype are avirulent on another and *vice versa*. We have yet to investigate this across a wide range of host and parasite genotypes in *P. chabaudi*, but in one experiment, the relative virulence of two parasite lines was never reversed on any of three mouse genotypes (Mackinnon et al. 2002). A cost of virulence one might expect, at least following serial passage, is loss of transmission-stage production (Ebert 1998). This has sometimes been seen in malaria parasites (Day et al. 1993), but in all our experiments to date, virulence increases have been associated with increased transmission-stage production (Mackinnon and Read 1999b; Mackinnon et al. 2002).

Taking all this together, we favor the hypothesis that host death imposes significant selection against pathogen virulence, and we assume that in what follows. But exactly why malaria parasites, or any other pathogens, are as benign as they are is a key unsolved problem in parasite evolution. We know of no compelling quantitative data demonstrating, for any pathogen, the factor(s) that prevent pathogens from being more virulent.

VACCINATION AND VIRULENCE EVOLUTION: THEORY

Here we consider the consequences of widespread vaccination of a population harboring a pathogen whose virulence is determined by the balance of the cost of virulence (host death) and the benefits (less rapid clearance by the host, enhanced ability to exploit host resources for transmission). We are envisaging that the vaccine is imperfect, in that it allows at least some transmission from some vaccinated hosts. Vaccines against some acute childhood infections can generate lifelong sterilizing immunity, but vaccines against other diseases do not (Plotkin and Orenstein 1999). In general, vaccines elicit levels of protection that are the same or worse than that elicited by natural infections. Many of the vaccines currently under development are attempting to elicit protection against diseases for which natural immunity is rather poor. In the absence of technological advances that endow new-generation vaccines with protective effects better than those achieved by nature, future vaccines will reduce disease levels but will not prevent some transmission from at least some vaccinated individuals. This is certainly likely for malaria vaccines. Natural immunity seems to wane rapidly and a high degree of immunity seems to require repeated exposure to parasites (Bruce-Chwatt 1963; Day and Marsh 1991; Marsh 1992; Richie and Saul 2002). Thus, it seems probable that, at best, levels of protection as modest as those seen naturally will be achieved by malaria vaccines. Indeed, malaria vaccines that have achieved statistically significant protection have done so by delaying rather than preventing the invasion of parasites or the onset of symptoms (Bojang et al. 2001; Genton et al. 2002). For the same reason, vaccines against other childhood diseases and those still under development, such as those against HIV or Hepatitis B and C, will be similarly imperfect or 'leaky.' Even some of the vaccines against childhood diseases may be very imperfect. For instance, vaccination against *Bordetella pertussis* has been staggeringly successful at reducing childhood deaths due to whooping cough, but the immunity elicited by the vaccine wanes within a few years. Consequently, the pathogen still circulates, even in populations with high levels of vaccine coverage (Ewald 1996).

Vaccines that impact some aspect of pathogen fitness without reducing it to zero have the potential to impose strong selection. The most obvious impact is selection for epitope mutants – variants that are able to at least partially evade vaccine-induced immunity. But there could also be potent selection on parasite virulence: vaccines have the potential to reduce the selection against virulent parasites. Vaccines that protect against host death also protect more-virulent pathogens from killing

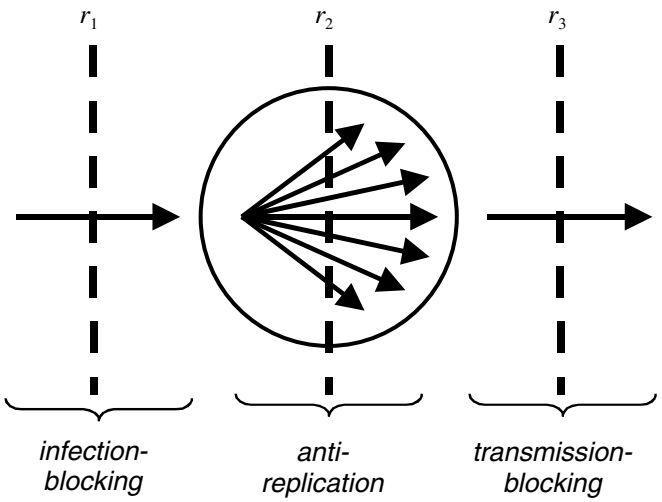

Figure 11.4. Schematic representation of the action of different types of host resistance at different stages of a parasite life cycle: r_1, infection-blocking resistance; r_2, anti-replication resistance; r_3, transmission-blocking resistance. A fourth type of resistance, r_4, anti-toxin resistance, prevents host death without impacting pathogen replication or transmission. Each of these four types of resistance is the target of various candidate malaria vaccines (Richie and Saul 2002): r_1, sporozoite and liver-stage vaccines; r_2, blood-stage vaccines; and r_3, transmission-blocking vaccines. Recently, a candidate r_4 vaccine has been proposed (Schofield et al. 2002). Figure is from Gandon et al. (2001) with permission.

their host and therefore themselves. Given the fitness benefits of virulence (transmission), more-virulent strains will then circulate in the population. Key here is the mode of action of the vaccine (Figure 11.4). In general, imperfect infection-blocking or transmission-blocking vaccines will not favor virulence increases because they do not alter the balance between the cost (mortality) and benefit (transmission) of virulence. In contrast, imperfect anti-disease vaccines (those reducing in-host replication or parasite toxicity) will always select for more-virulent pathogens: they protect both the host and the parasite from the costs of virulence. Elsewhere, we have formalized this verbal argument and we refer those interested in the mathematical details to that paper (Gandon et al. 2001). The argument is rather general and will also apply to any other medical intervention that reduces parasite replication or toxicity without eliminating the pathogens: subcurative chemotherapy and, in the veterinary context, enhanced genetic resistance through selective breeding will also select for more-virulent pathogens.

We are thus hypothesizing that anti-disease vaccines will prompt more-virulent pathogens to circulate in a population. Unvaccinated individuals, such as young children, travelers, and those unlucky enough not to get the vaccine, will therefore have a greater risk of contacting more-virulent pathogens and hence dying. If this argument is correct, it raises some interesting ethical issues. It is a quite different view of vaccine risk than that normally discussed in the tabloid press and public health circles. Normally, it is thought that, at no risk to themselves, unvaccinated individuals benefit through herd immunity from those who have been vaccinated and taken the (usually minute) risk of vaccine-induced side effects. Under our scenario, those who get the vaccine are putting at risk those who do not get the vaccine. We know of no other situation in which a medical intervention is beneficial for the recipient but makes things worse for those who do not receive it.

Our argument is rather general, though it obviously depends on a number of assumptions to which we return below. But it prompts additional questions, which can only be addressed with rather more disease-specific models. First, what is the population-wide disease burden? Even if unvaccinated individuals are at greater risk, the key question from the public health perspective is whether the population as a whole is better off after the vaccine-driven evolution has occurred. Unvaccinated individuals will be more likely to die, but the vaccine would not have been used at all if it did not reduce host death, lead to more rapid recovery, and/or make hosts less infectious. This is precisely what semi-immunity does in our mouse model (Buckling and Read 2001; Mackinnon and Read 2003). At a post-vaccine evolutionary equilibrium, how does population-wide death compare to the pre-vaccine case? Second, how rapid is this evolution likely to be? Clearly, if the time scale is measured in centuries or more, it need not be an issue for public health.

To answer these questions, we developed a malaria-specific population dynamic model in which virulence evolution was possible (Gandon et al. 2001). The model is a modified version of the standard susceptible–infected model, with two types of host (fully susceptible and semi-immune), and incorporates naturally acquired immunity, superinfection, and vector transmission. It was parameterized for a high-transmission year-round endemic *P. falciparum*. Broadly speaking, the above generalizations about the evolutionary consequences of different types of vaccine also hold for this model: infection-and transmission-blocking vaccines have minimal impact on virulence evolution, whereas vaccines targeted at malaria blood stages or parasite toxins are expect to prompt the evolution

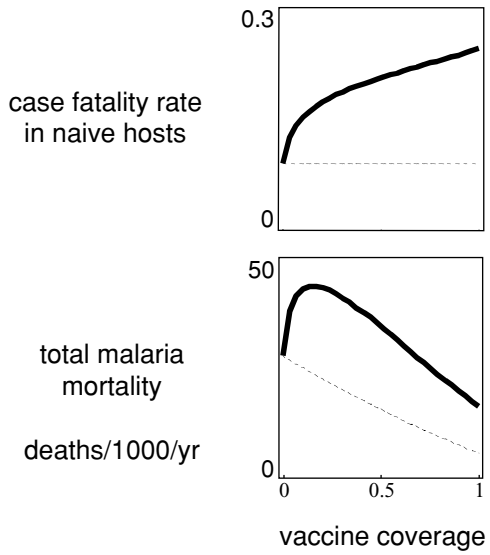

Figure 11.5. Predicted virulence in unvaccinated host and population-wide malaria mortality in a population vaccinated with an imperfect blood-stage (r_3) malaria vaccine. The dotted line indicates before evolution, as might be seen in a clinical trial or early in a vaccine campaign; the solid line indicates after evolution. For model details, see Gandon et al. (2001).

of more-virulent parasites. An example is shown in Figure 11.5. In the absence of pathogen evolution, pathogen virulence (here, case-fatality rate) is unaffected by vaccine coverage, but the population-wide deaths drop as vaccine coverage is increased. This is the conventional expectation, and what one would expect to see, for instance, in a clinical trial or early in the implementation of a vaccine program. However, vaccination protects virulent pathogens, so that they are expected to increase in frequency in the population. The post-vaccination equilibrium virulence is always expected to be higher, and the more people that are vaccinated, the higher it is. Consequently, case-fatality rates are higher among the unvaccinated. Among the population as a whole, the benefits of vaccination are eroded from those that would have been seen in the clinical trials or early in the implementation phase. There are even some regions of parameter space where the population as a whole is worse off than before vaccination (Figure 11.5). Moreover, once evolution has occurred, it would be difficult to halt vaccination even if death rates were similar or worse than the pre-vaccine era: withdrawal of the vaccine would put even more individuals at risk.

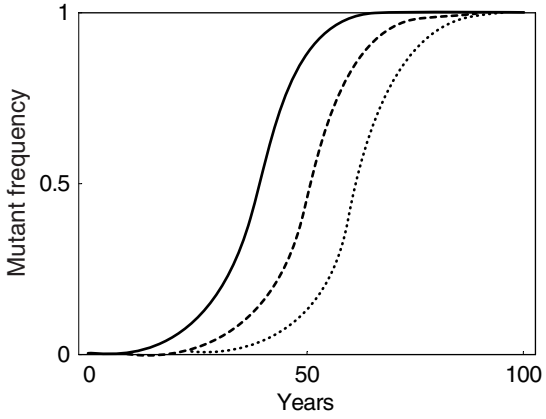

Figure 11.6. Invasion dynamics of a virulence mutant after the start of a vaccination campaign using an anti–growth rate vaccine ($r_2 = 0.8$ and $r_1 = r_3 = r_4 = 0$) with a vaccination coverage of 90%. In this simulation, the virulence of the resident strain is the ES virulence in the absence of vaccination ($\alpha_N = 0.153$). We present the invasion dynamics of invasion of a mutant with virulence equal to the ES level after vaccination ($\alpha^*_N = 0.418$). At the start of the vaccination campaign, we assumed that the mutant was at an initial frequency of 1% (solid line), 0.1% (dashed line), or 0.01% (dotted line). We further assumed that its distribution among naive and immune hosts was identical to the distribution of the resident strain before vaccination. For more details, see Supplementary Information in Gandon et al. (2001).

What of the time scale of this evolution? Predictions of rates of evolution are far more sensitive to specific model parameters and assumptions than are predictions about the direction of evolution or the magnitude of final equilibria. The time to fixation of variant alleles critically depends, for instance, on their initial frequency and selective advantage. In the current context, the selective advantage depends on the precise shape of the trade-off functions, and we simply know too little of the quantitative details to be sure of any estimates. Nonetheless, for plausible values, an anti–blood stage vaccine could drive virulence mutants to clinically relevant levels in a few decades (Figure 11.6). It is even possible that much more rapid evolution could occur: in some cases, epidemiological feedback on parasite evolution yields an evolutionary bistable situation where, for intermediate vaccine coverage, parasites can evolve toward high or low virulence, depending on initial conditions (Gandon et al., Personal Communication). Clearly, there is much more work to be done on the invasion dynamics of a virulence mutant, but from our simulations we expect that, as with the evolution of vaccine epitope escape mutants and drug resistance, the

evolution of virulence will occur on time scales relevant to public health (a few decades or even less).

VACCINATION AND VIRULENCE EVOLUTION: ASSUMPTIONS

Our conclusions about time scale, and the possibility that things can evolve to be worse than they were in the pre-vaccine era, are very parameter-(system) specific. However, the findings that anti-disease vaccines will prompt evolution that, at the population level, will erode the benefits of vaccines seen in clinical trials and early in implementation and that will put unvaccinated individuals at greater risk are very general conclusions that flow from our assumptions. The assumptions of our model that we see as key are as follows.

1. There is genetic variation in parasite virulence.
2. Vaccination is imperfect.
3. There is a positive genetic correlation between virulence and transmission.
4. The cost of virulence is host death.

Above, we reviewed the evidence relevant to these assumptions in the malaria context. Broadly speaking, the evidence is supportive; certainly we are unaware of any compeling contradictions from either animal experiments or the more anecdotal human data. Nonetheless, there are certainly a number of outstanding empirical issues. For instance, theory requires that the virulence–transmission relationship be saturating (that is, the marginal transmission advantages of virulence decrease as virulence increases): if it is not, selection would favor infinite virulence. We have yet to find direct evidence for saturation, although there are hints that it might be present (Mackinnon et al. 2002; Mackinnon and Read 2003) and theoretical reasons to expect it (Antia et al. 1994; Ganusov et al. 2002). There may also be other costs to virulence. For instance, extreme anemia may impact the parasites' ability to replicate. Also, genetically diverse infections are the rule rather than the exception in malaria (Day and Marsh 1991; Day et al. 1992), and we only modeled the case of superinfection (i.e. that in which hosts can be infected by more than one clone, but if a new clone establishes then it completely replaces the previous clone, so coexistence does not occur). Superinfection and coinfection can have profound impacts on the direction of virulence evolution depending on the mechanisms of any interactions between competing genotypes and how competitive outcome within hosts translates into between-host fitness (Read and Taylor 2001; Read et al. 2002). We are pursuing these issues

in our rodent model; so far generalities have proved elusive (Taylor et al. 1997; Taylor and Read 1998; Read and Taylor 2001; Mackinnon et al. 2002; Read et al. 2002; de Roode et al., submitted).

The extent to which these outstanding issues undermine our argument that blood-stage and toxin vaccines will select for more-virulent malaria parasites is unclear. We view absence of evidence as an opening for further work and understanding rather than as a fatal flaw (cf. Ebert and Bull 2003). If, for instance, other costs of virulence are discovered, these will need to be explored theoretically. If anemia is a cost of virulence, vaccination may reduce this too, with the same consequence for the evolution of malaria virulence. More telling empirical tests of the idea may also be possible. For instance, it should be possible to experimentally evolve malaria parasites in semi-immune and immunologically naive mice and compare the resulting virulence.

OTHER DISEASES

Of course, the real test of our hypothesis is the outcome of real-time evolution in human populations. Yet any evolutionary consequences of malaria vaccines are unlikely to become apparent during our working lives: even if the malaria vaccines currently under field trial actually work, it will be at least another 10–15 years before they could emerge from the regulatory morass of Western medicine and begin to impose selection on *Plasmodium* populations. Once they do, there will be no turning back. So what about diseases other than malaria? In many instances, vaccination has been going on for many decades, probably long enough for virulence evolution to occur. There is an urgent need to determine whether it has occurred or is occurring.

However, this will not be straightforward. Although the assumptions underpinning our model are likely to apply in many cases, they will certainly not apply to all diseases. For instance, we assume that virulence and transmission will be positively and genetically correlated. This is believed to be so in many but not all infectious diseases (Lipsitch and Moxon 1997; Weiss 2002; Ebert and Bull 2003). For instance, it is frequently stated that polio virus causes disease only when it gets into neural tissue, from which it has no transmission potential, and hence that there can be no link between virulence and transmission (Levin and Bull 1994). Actually, in this particular case we are not convinced. The reversion of the attenuated vaccine strains to wild type virulence in some parts of the world points to some sort of fitness advantage to virulence (Kew et al. 2002; Day, Personal Commnication) Nevertheless, the general point must be sound: virulence and transmission need not be genetically correlated. Clearly, our analysis

applies only to those situations in which they are: an absence of virulence evolution in a disease without such a correlation casts no light on our argument. We note, however, that even for *Plasmodium chabaudi* in laboratory mice, it has been an experimental challenge to determine relevant fitness functions. Where experimental work is not possible (human diseases without an appropriate animal model), it is going to be very difficult to determine whether a particular disease is of the sort that is likely to be in accord with our model.

Even if virulence evolution has occurred, it might be a serious challenge to detect it against other changes, especially where vaccination is highly effective. For illustrative purposes, consider measles in England and Wales, where vaccination began in the late 1960s. The best test of whether contemporary measles is more virulent than measles in the pre-vaccine era is to take strains from the 1960s and current strains and compare their virulence in an experimental common garden. This is how the evolution of the virulence of myxoma virus in Australia has been elucidated (Fenner and Fantini 1999). However, in the case of a human disease, there is no ethically acceptable common garden. One is forced to compare strains using a surrogate measure of virulence (such as *in vitro* growth rates, or virulence in an animal model) or to compare case records from the 1960s with contemporary disease outcome. Such a comparison would be hard enough, given undoubted changes in societal health generally and improvements in intensive and palliative care. This comparison becomes even more problematic when vaccination has reduced serious cases to very low numbers (as with measles in England and Wales), so that sample sizes become a problem. It may also be that many of the current UK cases are from pathogens initially contracted in populations in which there has been little or no vaccination.

An altogether more worrying prospect is that very large increases in virulence are in fact about to occur but we have yet to detect the early signs. As drug resistance has demonstrated, novel alleles rising from very low frequencies can be extremely hard to detect during the early part of their expansion, but then once they are at detectable levels, they very rapidly rise toward fixation to become a public health disaster (Hastings and D'Alessandro 2000). Chloroquine resistance in malaria, for example, must have first arisen in the 1950s but was not a serious problem until it became widespread two or three decades later. The situation could be even more difficult with virulence: drug resistance is a relatively uncontroversial phenotype to assay both *in vitro* and *in vivo*. In the absence of a genetic marker of virulence, it will be very, very difficult to detect changes in pathogen virulence until the evolution has proceeded a long way.

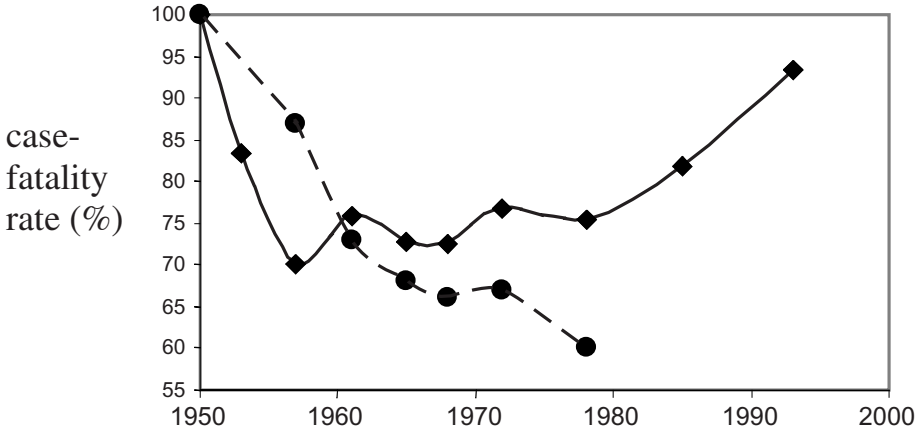

Figure 11.7. Myxomatosis virulence and rabbit resistance in Australia. Myxoma virus evolution was initially towards lower virulence, but virus virulence is increasing again (solid line, diamond), evidently in response to increases in resistance in wild rabbits (broken line, circles). Virulence of myxoma virus was estimated from mean survival times of wild isolates in a standard laboratory strain of rabbit; rabbit resistance was estimated by inoculating wild rabbits with a standard laboratory myxoma virus strain. Data are from Tables 14.5 and 14.6 of Fenner and Fantini (1999).

All of these issues make it difficult to test our hypothesis using human data in the absence of genetic markers. Nonetheless, we believe there is a strong case for trying to do so: we know of two examples of animal diseases that may be consistent with our proposed scenario. The first is myxomatosis in Australian rabbits. The dramatic drop in virulence that occurred a few years after the introduction of the myxoma virus in the 1950s is a textbook example of virulence evolution and one of the empirical examples used to support the argument that evolution favors intermediate levels of virulence that balance the benefits (high transmission and slower clearance) and costs (host death) of virulence. However, the implications of the more recent evolution of the myxoma virus have been largely overlooked (Gandon and Michalakis 2000). The virulence of isolates recovered from the field has been rising again (see Figure 11.7). In fact, more than 80% of the isolates were ranked as the most virulent strain. This percentage is the same as in the virus population introduced into Australia in the first place. The explanation for this seems to be increasing resistance in the rabbit population as a result of the strong natural selection imposed by the virus on the rabbit (Fenner and Fantini 1999). Studies of the infection process demonstrate that natural rabbit resistance has impaired the expansion of the virus population within the host (Best and Kerr 2000).

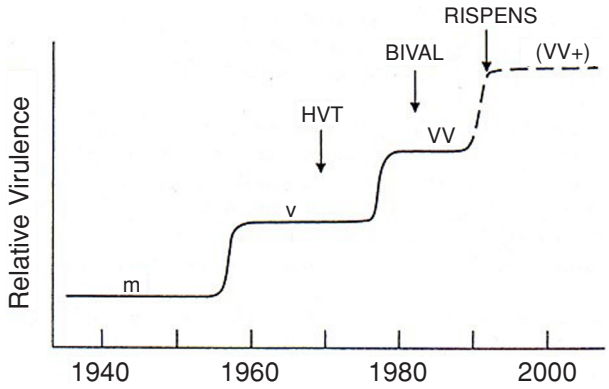

Figure 11.8. Schematic representation of Marek's disease virus virulence evolution, taken from the poultry literature. Until the 1950s, Marek's disease was caused by strains now classified as mild (m). These were then replaced by virulent (v), very virulent (vv), and very virulent + (vv+) strains. This evolution prompted the development of first-generation vaccines (HVT) and then second-(Bival) and third-(Rispens) generation vaccines. The original caption to this figure reads, "There appears to be a relationship between the introduction of new vaccines and the development of more virulent pathotypes" (Witter 1998, p. S50). Figure is from Witter (1998) with permission.

Thus, it is analogous to the resistance elicited by an anti-replication vaccine (r_2 in Figure 11.4). There is also evidence that more-virulent strains transmit better in the absence of host death (May and Anderson 1983; Best and Kerr 2000). Thus, it is most probable that increases in resistance are responsible for increases in virus virulence.

A possible case of vaccine-driven evolution is that of Marek's disease virus (MDV), which is a lymphoproliferative avian herpes virus that causes substantial losses in the poultry industry. There is extremely good evidence for strain differences in virulence, and this seems to have been the raw material that fueled substantial virulence evolution (Witter 1997a,b, 1988, 2001; Kreager 1998; Biggs 2001). From its first description in 1907 through to the middle part of the twentieth century, Marek's disease was a mildly paralytic syndrome associated with gross enlargements of peripheral nerves and occasional lymphomas. Death was relatively rare. This suite of symptoms was caused by strains now categorized as mild Marek's disease virus, mMDV. From the 1950s on, there has been a steady increase in virulence (Figure 11.8), and today hyperpathogenic strains dominate the poultry industry. These strains can kill within two weeks and are associated with syndromes such as flaccid neck paralysis and extensive lymphomas

on most internal organs. Direct comparisons of isolates from the past 50 years are not possible, but there seems little doubt that virulence evolution has occurred: mild MDV strains can no longer be isolated in commercial operations in the U.S., and it is difficult to imagine that hyperpathogenic strains could have been present at the end of World War II without someone recording the horrendous symptoms they cause.

What drove this evolution? Initial increases in virulence, attributed to the intensification of the poultry industry, prompted the development of the first generation of live attenuated vaccines (see Figure 11.8). This was shortly followed by further increases in virulence, which prompted the development of second-generation vaccines, more virulence, and subsequently the development of a third generation of vaccines. Many in the industry consider that the vaccines last about ten years and that virulence evolution has made vaccination an extremely fragile means of MDV control (Kreager 1998; Witter 2001): the next generation of vaccines may themselves be untenably virulent.

But is there a causal link between vaccination and the virulence increases? Certainly, this is a prevalent view in the poultry literature (Witter 1997a, 1998, 2001; Kreager 1998; Biggs 2001). A number of lines of evidence show that MDV accords with our model. First, there is good experimental evidence that vaccination is less protective against more-virulent strains (Witter 1997b); Read et al. in preparation). Second, the more-virulent strains are not vaccine escape mutants: there is absolutely no evidence that they are antigenically different from the milder ancestral strains. Instead, enhanced virulence is associated with enhanced proliferative ability; it looks very much like the sort of life-history (virulence) variation we are discussing. Third, the effects of the vaccine are anti-proliferative – r_2 in our speak (Figure 11.4). The vaccines may also have a pure anti-disease effect analogous to our r_4 resistance (Biggs 2001). Fourth, vaccinated birds transmit wild type virus, so the vaccine is imperfect. However, there are no quantitative data on transmission, so although a positive genetic correlation between virulence and transmission seems likely (pathogen-induced host cell proliferation is associated with both), it has not been confirmed.

It is of course possible that changes in factors other than vaccination efficacy were responsible for the virulence increases in MDV populations. One possibility is genetically enhanced resistance in the chickens. This resistance also slows virus proliferation, which would reinforce our argument that anti-replication immunity can drive virulence increases. Another possibility is that there have been other changes in the poultry

industry, not least intensification. Such hypotheses do not readily explain why more lethal strains are better able to cope with vaccination. To us, the lesson of Marek's disease is two-fold:

(1) virulence evolution on short time scales can have a huge impact on animal health, and
(2) vaccination might be the cause. There is an urgent need to determine whether it is, and if so, whether vaccination could have the same effect in diseases of humans.

ANTI-TOXIN VACCINES

Several authors have argued that there is evidence that anti-toxin vaccines have altered the virulence of at least one human disease (Ewald 1994, 1996, 2002; Soubeyrand and Plotkin 2002; Ebert and Bull 2003). However, the orthodoxy is that vaccination prompted the evolution of *less*-virulent pathogens. The most widely cited example is the bacterial disease diphtheria, caused by *Corynebacterium diphtheriae*, although the argument has been extended to *Bordetella pertussis* (Ewald 1994, 1996, 2002; Soubeyrand and Plotkin 2002; Ebert and Bull in press) and *Hemophilus influenzae* (Ewald 1996). The orthodox explanation for the reductions in virulence is as follows. The pathogen produces a fitness-enhancing toxin, but producing the toxin is metabolically expensive. Thus, in an unvaccinated host, the tox^+ variants have a fitness advantage, but in vaccinated hosts, the tox^- forms have an advantage because they pay no metabolic cost for producing a toxin rendered useless by immunity. Thus, in a vaccinated population, tox^- forms dominate, so that overall virulence goes down. Deliberately targeting vaccines at toxins to induce this sort of evolution (the "virulence-antigen strategy") has been advocated as a practical example of virulence management (Ewald 1994, 1996, 2002; Ebert and Bull 2003).

This argument contrasts with our view that anti-toxin vaccines will protect the host and hence the toxin-producing strains from risk of death, so that higher levels of toxin production (and hence virulence) can be sustained. The two arguments differ in what is considered the cost of virulence – host death (Gandon et al. 2001) or metabolic costs of toxin production (Ewald 1994, 1996, 2002; Soubeyrand and Plotkin 2002; Ebert and Bull 2003). In a model incorporating both costs (Gandon et al. 2002), we showed that increased or decreased virulence could result, depending on the metabolic cost of toxin production and vaccine coverage efficacy (Figure 11.9). This raises the very interesting possibility of testable

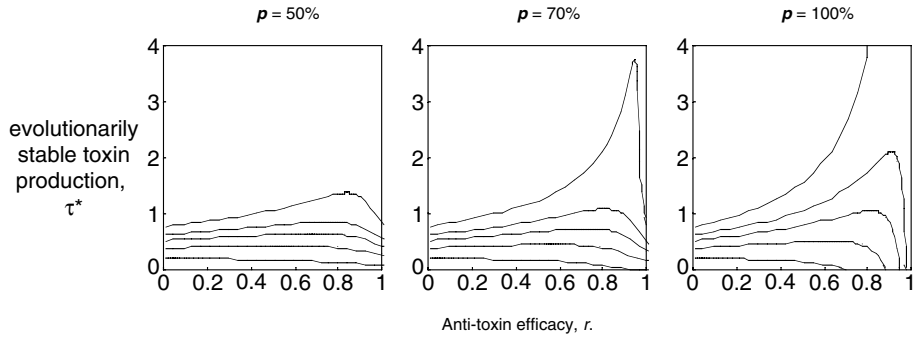

Figure 11.9. Evolutionarily stable toxin production, τ^*, against anti-toxin vaccine efficacy, r, for different toxin-production costs, c, and variable levels of vaccination coverage, p. We used the same fitness function and parameter values as Gandon et al. (2002) and the epidemiological model used here is equation (6) in Gandon et al. (2001).

predictions across a range of disease/epidemiological consequences and might provide a reason that, for instance, virulence reductions in diphtheria have apparently occurred in some contexts (e.g., Pappenheimer 1982), whereas there have apparently been increases in other populations (e.g., Mortimer and Wharton 1999). More generally, we believe our model to be the first formal attempt to model the virulence-antigen strategy; it is quite clear that it can have harmful evolutionary consequences under a fairly wide range of parameter space.

CONCLUDING REMARKS

We end with three points. First, there is absolutely no doubt that vaccination is one of the most important successes of biomedical science, and none of our data or theoretical models provides any argument against continuing to develop and implement mass vaccination. Even if we are right that *some* vaccines have the capacity to lead to the evolution of more-virulent pathogens, this is no argument for not developing them. Marek's disease is an important case in point. It may indeed be that vaccination is now responsible for the hyperpathogenic strains that now exist. But it has been estimated that vaccination has saved 2 billion chickens in the U.S. since its implementation (Witter 2001). Indeed, it is difficult to envisage any situation in which transitory benefits of disease relief for even just a few decades would not be worth having. For instance, malaria has a massive economic as well as human cost (Sachs and Malaney 2002). A

viable vaccine that reduced the malaria burden for even a few decades would liberate huge economic potential, given the nature of financial compounding. Economic development is a prerequisite for developing the health delivery infrastructure, implementing environmental measures, and improving housing, which could themselves have a huge impact on malaria incidence. *Thus, any malaria vaccine has huge potential for good*. Our strongest point is that, as with chemotherapy, we have to be aware that evolution can take the gloss off apparently magic bullets, so that we need to be vigilant and continually considering new control measures.

Second, our arguments are based on mathematical and animal models. For the most part, it is difficult to imagine any other way to investigate in advance the evolutionary safety of population-wide interventions in human pathogens. However, models of any sort rarely deliver certainty, and this necessarily means controversy. This is no reason to ignore the evolutionary consequences of vaccination. We suggest that more theoretical and empirical work of the sort we have been doing is called for on malaria and a range of other pathogens. Some of this work might serve to determine whether we are already unwittingly doing experiments on human populations that will have undesirable outcomes for which we should prepare. Other such studies might help us decide between possible candidate vaccines. For instance, transmission- and infection-blocking vaccines will generally lessen the risks of increased virulence and so should be incorporated in multi-component vaccines (Gandon et al. 2001). We also think that, during vaccine trials, substantially more attention should be paid to measuring transmission from vaccinated people. Finally, when vaccines go into widespread use, it is vital that extensive collections of frozen pathogen material be kept throughout, so that if evolutionary change does occur, it is possible to study it.

Finally, we believe that, at the very least, the Marek's disease story argues for more work on the impact of medical and veterinary interventions on virulence evolution. Something caused MDV to become more virulent over the past fifty years. Perhaps it was something other than vaccination, but it is surely some consequence of chicken husbandry. The poultry industry deals with virulence evolution by sterilizing empty chicken sheds. Analogous options are clearly unavailable to public health managers.

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