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Evaluation of the Effect of Host Immune Status on Short-Term *Yersinia pestis* Infection in Fleas With Implications for the Enzootic Host Model for Maintenance of *Y. pestis* During Interepizootic Periods

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Abstract

Plague, a primarily flea-borne disease caused by *Yersinia pestis*, is characterized by rapidly spreading epizootics separated by periods of quiescence. Little is known about how and where *Y. pestis* persists between epizootics. It is commonly proposed, however, that *Y. pestis* is maintained during interepizootic periods in enzootic cycles involving flea vectors and relatively resistant host populations. According to this model, while susceptible individuals serve as infectious sources for feeding fleas and subsequently die of infection, resistant hosts survive infection, develop antibodies to the plague bacterium, and continue to provide bloodmeals to infected fleas. For *Y. pestis* to persist under this scenario, fleas must remain infected after feeding on hosts carrying antibodies to *Y. pestis*. Studies of other vector-borne pathogens suggest that host immunity may negatively impact pathogen survival in the vector. Here, we report infection rates and bacterial loads for fleas (both *Xenopsylla cheopis* (Rothschild) and *Oropsylla montana* (Baker)) that consumed an infectious bloodmeal and subsequently fed on an immunized or age-matched naive mouse. We demonstrate that neither the proportion of infected fleas nor the bacterial loads in infected fleas were significantly lower within 3 d of feeding on immunized versus naive mice. Our findings thus provide support for one assumption underlying the enzootic host model of interepizootic maintenance of *Y. pestis*.

Keywords

Yersinia pestis; plague; flea; enzootic

Plague is a primarily flea-borne disease characterized by rapidly spreading epizootics in susceptible animal populations separated by periods of quiescence. Despite decades of research, however, it is still not clear how the etiologic agent of plague, *Yersinia pestis*, persists in endemic foci between outbreaks (Eisen and Gage 2009). A better understanding

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of the mechanisms underlying interepizootic maintenance of *Y. pestis* could aid in surveillance efforts and might make it possible to implement appropriate control measures between outbreaks, which could ultimately limit human exposure to the plague bacterium.

One long-standing hypothesis, referred to here as the enzootic host model, posits that *Y. pestis* persists between epizootics in a maintenance cycle involving fleas and relatively resistant enzootic hosts (Quan and Kartman 1962, Poland and Barnes 1979, Gage et al. 1995, Gage and Kosoy 2005, Buhnerkempe et al. 2011). According to this model, after an epizootic decimates a susceptible host population, infected fleas may infest a different species that includes both resistant (host survives infection) and susceptible (host dies after infection) individuals. When a resistant individual is infected with *Y. pestis*, it almost always seroconverts (Hudson et al. 1964, Levi et al. 1964, Chen and Meyer 1966). Thus, over time, the enzootic host population comes to include resistant individuals that at least temporarily carry antibodies to the plague bacterium, which can limit infection in the host following subsequent exposure to *Y. pestis* (Jawetz and Meyer 1943a,b). Studies suggest a relationship between antibody titers against *Y. pestis* fraction 1 (F1) antigen and immunity; a titer of ≥1:128 is protective in many species (Williams and Cavanaugh 1979, Centers for Disease Control and Prevention 1996). We refer here to those individuals within an enzootic population that have a protective antibody titer as “immune” hosts. During some periods, a significant proportion of the host population may fall into this category. In the 2-moperiod immediately after a 1962 epizootic in the San Francisco Bay area, for example, Hudson et al. (1964) detected antibodies against *Y. pestis* in 48% of trapped *Microtus californicus* (the California vole) and reported an average positive titer of 1:473.

Some have suggested that resistant animals might, for a short period, develop a sufficient bacteremia to infect feedings fleas before recovering (Goldenberg et al. 1964, Kartman et al. 1966). Recent analysis suggests, however, that to reliably infect feeding fleas, a host must develop a bacteremia of at least 10^6 colony-forming units (cfu)/ml (Engelthaler et al. 2000, Lorange et al. 2005). Such an overwhelming septicemia is likely to be fatal; Sebbane et al. (2005) detected bacterial concentrations between 10^4 and 10^7 cfu/ml in the majority of rats at the terminal stage of plague. Indeed, Eskey and Haas (1940) observed that it was possible to experimentally infect fleas by feeding them on infected guinea pigs only when the animals were within 42 h of death. Therefore, neither antibody-free nor antibody-carrying resistant animals that ultimately recover from an infection are likely to be infectious to feeding fleas. Within the enzootic host model, *Y. pestis* must therefore persist in a cycle between susceptible hosts and fleas. However, the number of susceptible individuals may be limited, and fleas must continue to take bloodmeals to survive. Presumably, resistant and immune hosts provide such bloodmeals, which dilutes the contact rate between infectious fleas and susceptible hosts (Eisen and Gage 2009). Thus, the heterogeneous (enzootic) host population endures and maintains *Y. pestis* long-term. Researchers have proposed a number of enzootic host species, including gerbils and marmots in Asia (Pollitzer and Meyer 1961, Gage and Kosoy 2005), the California vole and the deer mouse (*Peromyscus maniculatus*) in the western United States (Quan and Kartman 1962, Hudson et al. 1964, Poland and Barnes 1979), and field rodents (*Arvicanthis* and *Mastomys* spp.) in Kenya (Heisch et al. 1953).

Regardless of the species involved, the enzootic host model assumes that fleas remain infected after feeding on immune hosts. To our knowledge, no one has tested this assumption. Bell (1945) stated that a flea clears a *Y. pestis* infection more quickly after it feeds on an immune animal, but he did not cite any data or references. Other investigators have demonstrated that host antibodies can suppress infection with, or transmission of other pathogens in arthropod vectors (Azad and Emala 1987, Mendis et al. 1987, Fikrig et al. 1992, Gomes-Solecki et al. 2006). Within 4 d of feeding on vaccinated mice, for example, only 14% of ticks previously fed on mice infected with the Lyme disease spirochete remained infected compared with 80% of ticks fed on infected mice and subsequently fed on control mice (Fikrig et al. 1992).

Here, we undertook to test the hypothesis that feeding on an immune host will clear *Y. pestis* from an infected flea. Our study focused on *Xenopsylla cheopis* (Rothschild), the most important *Y. pestis* vector worldwide (Gratz 1999), and *Oropsylla montana* (Baker), a key *Y. pestis* vector in North America (Barnes 1982). During an interepizootic period, we would not expect a host population to be limited, and we would therefore expect a flea that has left an immune host to find another (potentially susceptible) host within a few days. Previous laboratory studies have shown that most *X. cheopis* and *O. montana* will readily take a new bloodmeal within 3 d of feeding (Eisen et al. 2006, 2007). Therefore, we sought to determine if a higher proportion of *Y. pestis*-infected fleas that subsequently fed on immunized mice would clear the infection within 3 d than infected fleas that were subsequently fed on naive mice. Because feeding on an immunized host might reduce but not clear an infection within 3 d, we also compared bacterial loads in fleas that remained infected after feeding on immunized versus naive mice.

Materials and Methods

Bacterial Strains

To determine whether or not fleas that have taken an infectious bloodmeal will remain infected after feeding on a host that has developed antibodies to *Y. pestis*, we first fed fleas on artificial feeders containing rat blood spiked with a fully virulent strain of *Y. pestis*, CO96-3188 (pMT1/pFra+, pCD1+, pPCP1+, Pgm+; LD_{50} of 10–100 cfu for mice; Engelthaler et al. 2000, Eisen et al. 2006). Two days postinfection, we allowed fleas to feed on a mouse that had been immunized with an attenuated strain of *Y. pestis* (immunized mouse-fed group), or an age-matched naive mouse (naive mouse-fed group). Mice were immunized with a live, attenuated strain (CO96-3188(Pgm−)) to simulate, as closely as possible, exposure to CO96-3188 in a naturally resistant host.

Pgm− *Y. pestis* mutants occur spontaneously with a frequency of $\approx 1 \times 10^{-5}$ (Brubaker 1969) and lack the 102-kb chromosomal *pgm* locus that includes the *Yersinia* high-pathogenicity island required for full virulence (Jackson and Burrows 1956, Buchrieser et al. 1998, Hinnebusch 2005). CO96-3188(Pgm−) had been previously generated in our lab by culturing *Y. pestis* CO96-3188 on Congo red agar and selecting white colonies (Perry and Bearden 2008). We verified the Pgm phenotype of both the wild-type and attenuated stocks used for this study by plating subcultures on Congo red agar (Surgalla and Beesley 1969). The presence or absence of the chromosomal *pgm* locus was further verified by polymerase

chain reaction amplification of the *hms*H gene, which lies within the *pgm* locus, using primers hmsH-F (5′-TGG CGG ATA CGC AGT ATG AC-3′) and hmsH-R (5′-GAC CCG CTT TAG ATT CTT CCT GTA-3′). A 5-min initial denaturation at 95°C was followed by 30 amplification cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, then a 10 min extension cycle at 72°C. We verified the presence of the three endogenous *Y. pestis* plasmids in both strains by plasmid profile analysis (rapid lysis method; Chu 2000) followed by electrophoresis at 4°C on a 0.6% agarose gel for 2.5 h at 100 V).

Inducing Immunity in Mice

We used an outbred murine model for this study because the immunogenicity of live, Pgm− *Y. pestis* mutant strains has been well-characterized in laboratory mice (Russell et al. 1995, Welkos et al. 2002). To induce an antibody response, we inoculated 6- to 8-wk-old Swiss Webster mice subcutaneously on day 0 with between 9.4×10^5 and 3.0×10^6 cfu of *Y. pestis* CO96-3188(Pgm−). Each mouse received booster inoculations of between 3.3×10^5 and 2.8×10^6 cfu on day 18 or 21 and again on day 32 or 35. Between 40 and 45 d post-initial inoculation we collected blood from each mouse and measured titers to the *Y. pestis* F1 antigen using passive hemagglutination and inhibition tests (Chu 2000). Only those immunized mice that achieved a titer of at least 1:128 were included in the study. We collected additional blood immediately after fleas fed on the mice (49–54 d post-initial inoculation), verified that we could not detect anti-F1 in the naive animals, and determined a feeding-day titer for each immunized animal.

Infecting Fleas

We used colony-reared *X. cheopis* or *O. montana* (Centers for Disease Control and Prevention Division of Vector-Borne Diseases, Fort Collins, CO) for all experiments. Fleas were infected via an artificial feeding system as described previously (Eisen et al. 2006). Briefly, mixed-age adult females were starved for 4–7 d, then allowed to feed on defibrinated Sprague–Dawley strain rat blood (Bioreclamation, Westbury, NY) spiked with 1.1×10^9 to 7.4×10^9 cfu/ml of *Y. pestis* CO96-3188. After 1 h, fleas were removed from the feeders, immobilized by chilling on ice, and examined by light microscopy for the presence of an obvious red bloodmeal in the midgut. Fed fleas were held at ≈23°C and ≈80% relative humidity(RH).

Flea Feeding on Immune or Naive Mice

Two day postinfection, batches of fleas were allowed to feed on anesthetized, immunized mice or on anesthetized, age-matched naive mice. After 1 h, fleas were removed from the mice and fed fleas were identified as described in Infecting Fleas section above. Fleas that had taken an infectious bloodmeal and subsequently fed on a mouse were held at ≈23°C and ≈80% RH for 3 d. All surviving fleas were then stored individually in microcentrifuge tubes at −80°C. All animal-handling procedures were approved by the Animal Care and Use Committee at the Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO.

Determining Flea Infection Rates and Bacterial Loads

To test for infection with *Y. pestis*, we macerated each flea in 90 µl of heart infusion broth (HIB) and 10 µl glycerol and plated 10 µl neat triturate or 100 µl of a 10^{-1} dilution of triturate in sterile saline on blood agar containing 6% sheep blood (SBA) or on a 1:1 mixture of *Yersinia* selective agar base and HIB supplemented with Bacto agar (CIN–HIB; Robinson et al. 2009). The latter was previously developed to encourage complete recovery of *Y. pestis* from flea triturates while restricting the growth of some other microbes (Robinson et al. 2009). Some triturates were plated on *Yersinia* selective agar with the addition of 1 µg/ml Irgasan. Fleas that tested negative on a selective medium were verified by replating on SBA before being scored as uninfected. Fleas that tested positive for *Y. pestis* infection via plating on any medium were scored as infected. Cultures that could not be easily identified as *Y. pestis* based on colony color and morphology were subcultured, incubated overnight at 37°C, suspended in saline, and tested for the presence of *Y. pestis* F1 via direct fluorescent antibody staining (Chu 2000).

A number of *X. cheopis* were contaminated with a swarming bacterium that made them impossible to quantify on SBA or CIN–HIB. This contaminant was identified as *Proteus* sp. by amplifying a segment of the 16S rRNA gene with eubacterial primers 63F and 1387R and sequencing the segment with primers 63F, 1387R, 101F, 537R, 519F, and 926R (Kugeler et al. 2005). Sequences were assembled using Lasergene (DNASTAR, Madison, WI), and we used the basic local alignment search tool to identify highly similar sequences in the nucleotide database. We found that *Yersinia* selective agar supplemented with 1 µg/ml Irgasan controlled *Proteus* sp. in contaminated flea cultures, but the *Y. pestis* CO96-3188 cfu counts on this medium were generally at least a log lower than those on SBA (data not shown). We therefore sought to identify an alternative medium for use in determining flea bacterial loads for this study. By serially diluting a *Proteus* sp. culture and plating it on heart infusion agar supplemented with 0.25 µg/ml Irgasan (HI + I), we verified that this selective medium almost completely inhibits *Proteus* sp. at concentrations $\leq 3.2 \times 10^7$ cfu per plate. Specifically, after incubating for 72 h at 28°C, we observed a single colony on one of the six HI + I plates seeded with 3.2×10^6 cfu *Proteus* sp., and no growth on any of the six HI + I plates seeded with 3.2×10^7 cfu *Proteus* sp. Plating serial dilutions of 20 contaminated *X. cheopis* triturates from this study revealed that none contained $>1.6 \times 10^6$ cfu *Proteus* sp. We therefore concluded that HI + I sufficiently controlled *Proteus* sp. growth to allow for quantification of our contaminated flea triturates. We verified that HI + I does not dramatically inhibit *Y. pestis* growth by plating serial dilutions of 24 uncontaminated *Y. pestis*-infected flea triturates in duplicate on HI + I and SBA. Cfu per flea calculations based on the HI + I colony counts were well within one log of those based on the SBA colony counts.

We determined bacterial loads for all fleas that tested positive for *Y. pestis* by serially diluting triturates in sterile saline and plating in duplicate on HI + I. Cultures were incubated at 28°C for 72 h and we calculated bacterial loads from cfu counts. Any infected flea that yielded <25 cfu per 100 µl of 10^{-1} dilution was considered to contain *Y. pestis* at numbers too low to quantify.

Statistics

The proportion of fleas infected with *Y. pestis* was compared between treatments (immunized mouse-fed vs. naive mouse-fed) using likelihood ratio tests (χ^2 values reported) and Fisher's exact tests (one-tailed *P* values reported). We compared bacterial loads between treatments using Mann–Whitney *U* tests with chi-square approximations (χ^2 and *P* values reported). We conducted all statistical analyses using JMP 10 statistical software (SAS Institute, Cary, NC). All statistical tests were considered significant at $P < 0.05$.

Results

In total, 18 of the 19 mice that received three inoculations with CO96-3188(Pgm−) achieved an antibody titer of at least 1:128 by the week preceding flea feeding and were included in the study (data not shown). Individual mouse titers on the day mice provided bloodmeals to fleas ranged from 1:128 to 1:2048 (Tables 1 and 2).

The proportion of *X. cheopis* that was infected with *Y. pestis* 5 d after consuming an infectious bloodmeal and subsequently feeding (2 d postinfection) on an immunized mouse (89.6% of 298 fleas) was similar to the proportion of *X. cheopis* that was infected after taking an infectious bloodmeal and subsequently feeding on a naive mouse (90.1% of 312 fleas; $\chi^2 = 0.036$; df = 1; $P = 0.48$; Table 1). Fourteen *X. cheopis* (eight immune mouse-fed and six naive mouse-fed) were excluded from all analyses because contamination with other microbes made it impossible to determine each flea's infection status on SBA or CIN–HIB and the bacterial load, if any, was too low to detect on *Yersinia* selective agar containing antibiotic. Even if all of the contaminated immune mouse-fed fleas were uninfected and all of the contaminated naive mouse-fed fleas were infected, however, the proportion of *X. cheopis* infected after feeding on an immunized mouse would still not be significantly lower than the proportion of *X. cheopis* infected after feeding on a naive mouse ($\chi^2 = 1.408$; df = 1; $P = 0.14$).

Likewise, the proportion of *O. montana* that was infected with *Y. pestis* after consuming an infectious bloodmeal and subsequently feeding on an immunized mouse (98.2% of the 165 fleas) was similar to the proportion of *O. montana* that was infected after taking an infectious bloodmeal and subsequently feeding on a naive mouse (100% of the 151 fleas; $\chi^2 = 3.925$; df = 1; $P = 0.14$; Table 2).

In total, 14 of the 267 (5.2%) *X. cheopis* that were still infected 3 d after feeding on immunized mice had bacterial loads too low to quantify, as did 12 of the 281 (4.3%) naive mouse-fed *X. cheopis*. The 253 immunized mouse-fed *X. cheopis* with quantifiable bacterial loads had a median of 2.20×10^5 cfu per flea. This was similar to the median for the 269 naive mouse-fed *X. cheopis* with quantifiable bacterial loads (2.30×10^5 cfu per flea; $\chi^2 = 0.0800$; df = 1; $P = 0.78$; Table 1).

One of the 162 (0.6%) infected immune-mouse fed *O. montana* contained too few *Y. pestis* to quantify, as did 3 of the 151 (2.0%) infected naive mouse-fed *O. montana*. Among the remaining infected *O. montana*, the median bacterial load in immunized mouse-fed fleas (1.26×10^5 cfu per flea) was higher than in naive mouse-fed fleas (6.10×10^4 cfu per flea;

$\chi^2 = 9.2255$; df = 1; $P = 0.0024$; Table 2). Although this difference was statistically significant, it should be noted that the two treatment groups did not have equal variances (Brown–Forsythe test, F ratio = 9.1908; $P = 0.0026$) and the difference between mean bacterial loads was less than a log.

Discussion

We did not find any evidence that feeding on an immunized host clears *Y. pestis* in *X. cheopis* or *O. montana*. While our study focused on short-term infection rates (the proportion of fleas infected 3 d after feeding on an immunized or naive host), analysis of bacterial loads in both *X. cheopis* and *O. montana* did not show significant reductions in cfu per flea in fleas fed on immunized versus naive mice. While we cannot rule out the possibility that clearance might occur at a later time point, there is nothing in our data to suggest that we would see more rapid clearance in fleas fed on immunized mice. This is particularly true within the time frame when a flea would be likely to find a new host during interepizootic periods when host abundance is not reduced by plague-induced mortality.

Thus, in contrast to the early assertions of Bell (1945) that feeding on immunized hosts clears infection, which would reduce the force of infection by reducing the abundance of infected fleas feeding on an enzootic host population, our findings support the relevant aspect of the enzootic host model as a viable hypothesis to explain interepizootic maintenance of *Y. pestis*. The finding that a bloodmeal from an immunized host does not reduce bacterial loads in *Y. pestis*-infected *X. cheopis* or *O. montana* within 3 d of feeding may be explained by the way in which *Y. pestis* colonizes the flea midgut. *Y. pestis* aggregates in the midgut are enveloped in a viscous extracellular matrix (Hinnebusch et al. 1996, Jarrett et al. 2004). This matrix might prevent anti-*Y. pestis* antibodies or other blood-associated factors from adhering to surface targets on aggregated *Y. pestis* cells in the flea midgut. Indeed, Jarrett et al. (2004) reported that anti-*Y. pestis* polyclonal antibody used in immunofluorescence antibody assays did not recognize the gut-associated matrix or *Y. pestis* enclosed within it.

We stress that this study addresses only one assumption underlying the enzootic host model. The viability of the enzootic host hypothesis depends on numerous factors, including transmission efficiency, infestation rates, and host feeding preferences (Eisen and Gage 2009). Other recent research has highlighted the need for further investigation of this hypothesis. For example, one study evaluated early-phase transmission efficiency of *Aetheca wagneri* (Baker), a predominant flea on deer mice, which are commonly considered likely plague reservoirs (Kartman et al. 1966, Poland and Barnes 1979), and showed that transmission efficiency was too low to support enzootic maintenance of *Y. pestis* unless flea loads were exceptionally high (Eisen et al. 2008b). Another field study showed that the dynamics of flea-sharing between deer mice and prairie dogs (*Cynomys ludovicianus*) did not support the hypothesis that deer mice serve as enzootic hosts (Salkeld and Stapp 2008).

While our results support one aspect of the enzootic host model, the finding that fleas can remain infected after feeding on immunized hosts is also consistent with the hypothesis that *Y. pestis* may persist in fleas. There is evidence that in some cases, an infected flea may

survive the winter season or longer and remain infected (Prince and Wayson 1947a, Kartman et al. 1962), and it may thus transmit the bacterium to a susceptible host many months after taking an infectious bloodmeal (Prince and Wayson 1947b). This inefficient transmission mechanism might allow *Y. pestis* to persist at low levels during interepizootic periods (Eisen and Gage 2009). This mechanism would likely require that infected fleas survive by feeding on hosts that have survived epizootics and therefore carry antibodies to the plague bacterium. The ability to maintain an infection after feeding on such hosts is therefore critical to the hypothesis that fleas may act as a *Y. pestis* reservoir.

Although our results support one aspect of the enzootic host model and are consistent with the flea reservoir hypothesis, it is worth noting several caveats to our experimental design. First, it is possible that we would have observed more clearing in the immunized host-fed fleas if we had held the fleas for >3 d after feeding on the mice. A previous study, however, detected host antibodies in the *X. cheopis* gut beginning 3 h after exposure to an immunized host, and the antibodies persisted in the gut for only 48 h after fleas were removed from the host (Azad and Emala 1987). We would therefore expect to see any antibody-mediated clearing within a few days of allowing the fleas to feed on immunized mice. It is possible that other flea- and blood-associated factors might impact flea infection over a longer period of time. We note, however, that Eisen et al. (2008a) reported significantly higher infection prevalence in *X. cheopis* and *O. montana* collected 48 h after feeding on *Y. pestis*-spiked rat blood than in fleas collected 48 h after feeding on spiked rabbit or mouse blood. This suggests that host blood-associated factors that can impact infection prevalence can have that impact within 2 d of feeding. In addition, as noted above, our bacterial load data did not indicate any decrease in *Y. pestis* cfu per flea values that might be associated with clearance over a longer period.

Second, we examined the infection status and bacterial loads in fleas 3 d after they had taken a single bloodmeal from an immunized or naive host. During an interepizootic period, an infected flea could be expected to take multiple subsequent bloodmeals from one or more hosts with an anti-*Y. pestis* titer. It is possible that taking multiple bloodmeals from an immune host or hosts could clear the infection.

Third, we immunized mice via needle inoculation with an attenuated *Y. pestis* mutant. We acknowledge that this may have elicited a somewhat different immune response than flea-mediated transmission of virulent *Y. pestis* to a resistant host. There may, for example, be flea-mediated transmission factors akin to those associated with tick and sandfly saliva, both of which contain immunomodulators and enhance pathogen infectivity to the vertebrate host (Titus et al. 2006). In addition, the attenuated strain against which the mice developed antibodies was not identical to the virulent strain with which we infected the fleas. Indeed, exposure to virulent *Y. pestis* may evoke a different immune response in a resistant animal than exposure to attenuated *Y. pestis* evokes in a susceptible one. Notably, however, the findings of Jawetz and Meyer (1943b) suggest that ground squirrels that survived inoculation with virulent *Y. pestis* and ground squirrels inoculated with similar numbers of attenuated *Y. pestis* bacilli were similarly protected against subsequent challenge with virulent *Y. pestis*. Furthermore, our inoculating strain, CO96-3188(Pgm−), contains both the pMT1–pFra and pCD1 plasmids, which carry the genes encoding F1 and V antigen,

respectively (Dennis and Mead 2010). Both F1 and V antigen consistently elicit a protective immune response against challenge with *Y. pestis* in mice (Titball and Williamson 2003). It is also true, however, that expression of F1 and of V antigen are upregulated at 37°C and there is little if any synthesis of these antigens at the ambient temperature of the flea (Perry and Fetherston 1997, Hinnebusch 2005), so antibodies against F1 and V antigen are unlikely to act directly on bacteria in the flea gut.

We also acknowledge that there may be host species-specific differences in the ability of serum components to kill *Y. pestis* in the flea gut. It is possible that a bloodmeal from a putative enzootic host species (e.g., the deer mouse or California vole) carrying antibodies to the plague bacterium might have a different effect on *Y. pestis* in the flea than a bloodmeal from an immunized Swiss Webster mouse. It is clear that inoculation with attenuated *Y. pestis* strains, including Pgm− mutants, can elicit protective immunity in laboratory mice (Jawetz and Meyer 1943b, Russell et al. 1995, Welkos et al. 2002). In one experiment, mice that had survived vaccination experiments with live, attenuated *Y. pestis* and were subsequently reinfected with 10^7 virulent *Y. pestis* organisms had no detectable bacteria in their blood 4 d postchallenge and only one of the eight mice tested had a detectable bacteremia between 2 and 3 d postchallenge. This was in contrast to naive controls, in which bacteria could be detected in the blood at least 7 d postinfection, suggesting that, at least in vivo, factors in immunized mouse blood are lethal to *Y. pestis* and act within a few days of infection (Jawetz and Meyer 1943a). It is also true, however, that researchers have reported differences between mouse serum and sera from other mammals in terms of anti-*Y. pestis* activity (Bartra et al. 2008). We cannot rule out the possibility that use of alternative hosts could alter our results.

Finally, it should be noted that an infected flea is not necessarily infectious; it is possible that taking a blood-meal from an immune host might decrease the probability of future transmission even though the flea remains infected. Azad and Emala (1987) reported that *X. cheopis* infected with *Rickettsia typhi* and subsequently maintained on immunized rats remained infected, but unlike infected fleas maintained on control rats, they failed to transmit the rickettsiae 19–22 d postinfection. Further investigation is needed to determine whether feeding on an immune host might impact subsequent *Y. pestis* transmission efficiency.

In conclusion, our study finds support for one assumption of the enzootic host model, but highlights the need for evaluation of other model assumptions in various enzootic host systems.

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Table 1

Proportion *X. cheopis* infected with *Y. pestis* and bacterial loads in fleas after feeding on artificial feeders containing defibrinated rat blood spiked with 1.4×10^9–7.4×10^9 cfu/ml *Y. pestis* (CO96-3188), taking a second bloodmeal 2 d postinfection from a mouse immunized with an attenuated *Y. pestis* strain (CO96-3188(Pgm−)) or from an age-matched naive mouse, and subsequently being held alive for 3 d

Immune status and mouse no.	Mouse titer	Percent fleas infected after feeding on mouse (no. infected/no, fed on mouse)	Median (range) cfu per flea in fleas with a quantifiable bacterial load
Immunized mice			
1	1:128	97.5% (39/40)	5.80×10^4 (6.15×10^2 to 2.37×10^6); $n = 35$
2	1:256	88.5% (23/26)	2.18×10^5 (1.50×10^3 to 1.83×10^6); $n = 22$
3	1:512	82.4% (14/17)	1.70×10^5 (1.33×10^3 to 1.44×10^6); $n = 14$
4	1:512	87.5% (14/16)	4.05×10^5 (1.47×10^3 to 1.89×10^6); $n = 13$
5	1:512	90.5% (19/21)	3.65×10^5 (4.86×10^2 to 1.76×10^6); $n = 19$
6	1:1024	73.3% (11/15)	2.17×10^5 (1.02×10^4 to 1.87×10^6); $n = 11$
7	1:1024	100% (19/19)	1.54×10^6 (9.05×10^4 to 3.86×10^6); $n = 19$
8	1:1024	84.6% (33/39)	5.05×10^4 (6.70×10^2 to 1.34×10^6); $n = 30$
9	1:1024	92.9% (39/42)	1.89×10^5 (4.75×10^2 to 1.48×10^6); $n = 39$
10	1:1024	83.8% (31/37)	2.75×10^4 (3.55×10^2 to 1.68×10^6); $n = 27$
11	1:2048	96.2% (25/26)	1.08×10^6 (1.16×10^4 to 3.01×10^6); $n = 24$
Naive mice			
1	0	83.3% (10/12)	5.58×10^5 (1.45×10^4 to 1.28×10^6); $n = 10$
2	0	94.4% (17/18)	2.70×10^5 (3.90×10^2 to 1.58×10^6); $n = 17$
3	0	87.5% (14/16)	4.28×10^5 (7.05×10^3 to 1.28×10^6); $n = 14$
4	0	100% (25/25)	6.10×10^5 (4.45×10^4 to 2.07×10^6); $n = 25$
5	0	100% (22/22)	4.60×10^5 (8.55×10^2 to 1.95×10^6); $n = 22$
6	0	94.4% (17/18)	1.00×10^6 (1.77×10^5 to 1.63×10^6); $n = 17$
7	0	90.0% (18/20)	7.60×10^5 (1.66×10^4 to 3.05×10^6); $n = 18$
8	0	100% (17/17)	6.50×10^5 (1.09×10^3 to 2.43×10^6); $n = 17$
9	0	92.9% (39/42)	5.60×10^4 (3.90×10^2 to 2.68×10^6); $n = 37$
10	0	85.7% (30/35)	7.45×10^4 (3.50×10^2 to 1.22×10^6); $n = 28$
11	0	82.2% (37/45)	8.30×10^4 (1.46×10^3 to 1.20×10^6); $n = 35$
12	0	83.3% (35/42)	3.80×10^4 (4.05×10^2 to 6.95×10^5); $n = 29$

Table 2

Proportion *O. montana* infected with *Y. pestis* and bacterial loads in fleas after feeding on artificial feeders containing defibrinated rat blood spiked with 1.1×10^9–3.1×10^9 cfu/ml *Y. pestis* (CO96-3188), taking a second bloodmeal 2 d postinfection from a mouse immunized with an attenuated *Y. pestis* strain (CO96-3188(Pgm−)) or from an age-matched naive mouse, and subsequently being held alive for 3 d

Immune status and mouse no.	Mouse titer	Percent fleas infected after feeding on mouse (no. infected/no, fed on mouse)	Median (range) cfu per flea in fleas with a quantifiable bacterial load
Immunized mice			
1	1:128	94.7% (18/19)	1.26×10^5 (3.10×10^3 to 1.95×10^6); $n = 18$
2	1:512	100% (10/10)	8.93×10^4 (5.45×10^3 to 1.81×10^6); $n = 10$
3	1:512	100% (10/10)	1.92×10^5 (5.50×10^3 to 1.80×10^6); $n = 22$
4	1:512	96.6% (28/29)	1.08×10^5 (3.65×10^3 to 6.75×10^5); $n = 28$
5	1:512	100% (33/33)	6.48×10^4 (1.47×10^3 to 8.05×10^5); $n = 32$
6	1:1024	95.5% (21/22)	4.75×10^4 (4.20×10^3 to 1.87×10^6); $n = 21$
7	1:1024	100% (30/30)	9.50×10^4 (6.00×10^2 to 1.05×10^6); $n = 30$
Naive mice			
1	0	100% (21/21)	5.25×10^4 (4.70×10^2 to 1.95×10^6); $n = 21$
2	0	100% (16/16)	5.85×10^4 (1.32×10^3 to 1.52×10^6); $n = 15$
3	0	100% (20/20)	7.20×10^4 (4.45×10^3 to 1.85×10^5); $n = 20$
4	0	100% (22/22)	5.28×10^4 (2.00×10^3 to 9.25×10^5); $n = 22$
5	0	100% (15/15)	4.03×10^4 (5.20×10^3 to 5.15×10^5); $n = 14$
6	0	100% (26/26)	1.01×10^5 (3.45×10^3 to 3.95×10^5); $n = 26$
7	0	100% (31/31)	3.45×10^4 (1.14×10^3 to 3.95×10^5); $n = 30$