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Predicting the Outcome of Infectious Diseases: Variability among Inbred Mice as a New and Powerful Tool for Biomarker Discovery

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ABSTRACT Individuals respond differently to infectious diseases. Even among inbred mice that are presumed to be genetically identical, the response to a microbial pathogen is variable, which is generally thought to reflect experimental inconsistencies, technical errors, and stochastic processes. Here we describe the remarkable observation that the variability of *Helicobacter pylori* colonization density in the stomachs of experimentally infected C57BL/6J mice is tightly correlated with weight loss and viral load after a challenge with influenza virus, though *H. pylori* infection *per se* does not affect influenza and vice versa. Since these two infectious agents are found in different tissue compartments and are detected using unrelated methods, the correlation in microbial burden must represent a biological measure of disease susceptibility among genetically nearly identical individuals and not technical or stochastic factors. We hypothesize that inbred mice represent a powerful new tool for the identification of biomarkers to predict the outcome of infectious diseases.

THE OBSERVATION: VARIABILITY IN THE OUTCOME OF EXPERIMENTAL INFECTION AMONG INBRED MICE IS NOT STOCHASTIC

Individuals respond differently to infectious diseases; a particular microbe might cause serious disease in one individual but only a mild or even subclinical infection in another. Many factors contribute to such differences, most notably, the dose and route of inoculation, host and pathogen genetics, pre-existing immunity, and concurrent infection with mutualistic species or other pathogens. In humans, each of these factors is uncontrolled and often unknown, so differences in the response to infection are not surprising. Animal models, particularly inbred mice, are used to overcome these differences. Individual inbred mice of the same strain and from the same breeding colony are usually considered to be genetically identical. When they also share a microbiome because they are from the same litter and are cohoused, they should, in principle, respond identically to experimental infection.

Yet even when clonal pathogens are administered to inbred mice, differences in outcome are common. In our laboratories, where we study influenza virus and the gastric pathogen *Helicobacter pylori*, we commonly find marked differences in the microbial burden after experimental infection. For example, inbred female C57BL/6J mice of identical age, obtained from The Jackson Laboratory and maintained in microisolator cages under identical conditions, show as much as a 1,000-fold range of lung viral loads at the height of infection with influenza A virus (Fig. 1A). Similarly, infection of female, age-matched C57BL/6J inbred mice with *H. pylori* strain PMSS1, which readily colonizes and induces chronic gastritis in mice (1), results in variable bacterial colonization levels (Fig. 1B). Similar observations are widely reported by others and are generally thought to reflect stochastic effects, measurement errors, or imperfect control of experimental variables but not biologically meaningful differences.

Surprisingly, however, when we infected mice first with *H. pylori* and then 2 months later with influenza A virus, *H. pylori* colonization densities in the stomachs of individual mice showed a strong positive correlation with the influenza viral loads in their lungs (Fig. 1C; $R^2 = 0.89$, $P = 0.005$). In other words, knowledge

of the *H. pylori* bacterial load in the stomach could have predicted the influenza viral load in the lung. Weight loss is an indication of influenza severity in mice and is correlated with viral titers (Fig. 2A; $R^2 = 0.38$, $P = 0.03$). Interestingly, the *H. pylori* burden was also highly correlated with weight change after influenza virus infection and, in fact, was a better predictor than the influenza virus titer (Fig. 2B; $R^2 = 0.53$, $P = 0.007$). Thus, the bacterial load of *H. pylori* in the stomach predicts both virologic and clinical outcomes of influenza virus infection among inbred mice. Together, these data suggest that individual inbred mice differ in the capacity to control disparate infections in two distinct tissue compartments.

Although differences in susceptibility to infection between mouse strains have long been appreciated and even used to identify host immune factors and functions (summarized in reference 2), our work suggests that individual mice of a single inbred strain exhibit sufficient phenotypic variability to affect the severity of disease. Others have similarly reported striking differences in the outcome of infections within groups of inbred mice. For example, ~ one-quarter of age- and sex-matched mice infected with *Salmonella enterica* serovar Typhimurium become “supershedders” that are competent for transmission (3). Similarly, only a subset of inbred mice infected with uropathogenic *Escherichia coli* develop persistent bacteriuria, rather than resolve the infection, and these two groups of mice have distinct cytokine profiles in response to infection (4). Yet even these marked differences in the host response to infection among inbred mice of the same strain are commonly attributed to experimental error, colonization bottlenecks that result from stochastic processes (4), or differences in

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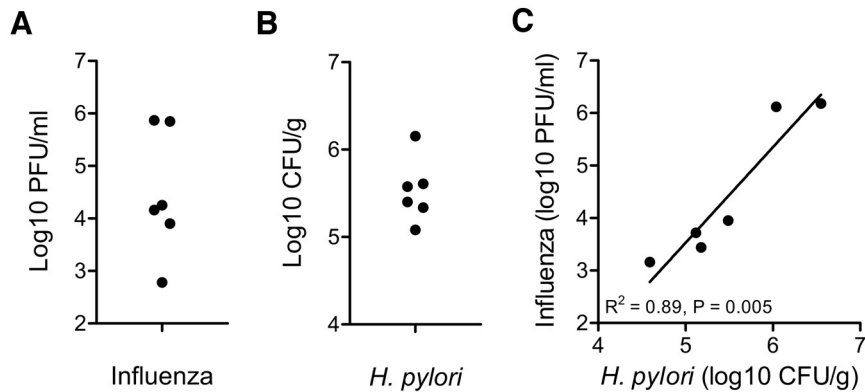


FIG 1 The microbial burdens of *H. pylori* and influenza virus are variable in singly infected C57BL/6 mice but show a strong positive correlation in coinfecting mice. (A) Female age-matched C57BL/6J mice were challenged intranasally with 20 PFU of influenza A/Puerto Rico/8/34 virus (A/PR8) and sacrificed 6 days later. Lung colonization was determined from whole lung homogenate by quantitative PCR. (B) Female age-matched C57BL/6J mice were infected singly by gavage with 2.5×10^9 CFU of *H. pylori* PMSS1 and sacrificed 8 weeks later. The gastric colonization (number CFU per gram) of each mouse was determined by plate counts on selective medium. *H. pylori* and influenza virus microbial burdens among singly infected mice varied by 10- to 1,000-fold, respectively. (C) *H. pylori* bacterial loads and influenza viral loads were determined in age-matched female C57BL/6J mice challenged with influenza A virus 8 weeks after infection with *H. pylori* PMSS1 as described above. Linear regression analysis showed a highly significant positive correlation between *H. pylori* loads and influenza virus titers. Each symbol represents a value from an individual mouse.

microbiota (3). Here we propose the alternative hypothesis that they may, in fact, represent subtle biological differences that alter the response to infection in an individual mouse.

THE MECHANISM: WHAT MIGHT EXPLAIN DIFFERENCES IN INFECTIOUS DISEASE OUTCOMES AMONG INBRED MICE?

What is the molecular basis for the variability in disease outcome and infectious burden among inbred mice following infection with a given pathogen, and what mechanisms could explain the correlation between *H. pylori* colonization levels in the stomach and influenza viral loads in the respiratory tract of the same animal? While the variability in microbial burden with one pathogen could be dismissed as technical or experimental error, the corre-

lation between the two pathogens strongly suggests biological differences among C57BL/6J mice. The most straightforward explanation would be that one infection (*H. pylori*) predisposes or protects from infection with the other (influenza virus). Indeed, mounting data suggest a correlation between the composition of the gastrointestinal microbial community and immune responses, including those to influenza virus (5). We initially predicted such an effect for our coinfection experiments with *H. pylori* and influenza virus. However, *H. pylori* did not affect influenza viral titers (Fig. 3A) or influenza-induced weight loss (Fig. 3B). Furthermore, bronchoalveolar lavage fluid collected from mice infected with influenza virus had similar concentrations of 32 different cytokines and chemokines (Millipore Milliplex MAP Mouse Cytokine/Chemokine Panel), regardless of whether the mice had been inoculated with *H. pylori* or with a broth control (data not shown). Similarly, the density of *H. pylori* colonization in the stomach was unchanged by subsequent infection with influenza virus (Fig. 3C). Together, these results suggest that the immune response to influenza virus or *H. pylori* does not affect host control of the other in this mixed-infection model and that the explanation for our results lies elsewhere.

Differences in the outcome of infection might result from variability in gut microbiota, which is well known to affect the response to microbial pathogens (6). Although not tested experimentally, this is an unlikely explanation for our observations, since the mice were purchased from the same vendor, were the same sex and age, and were cohoused under identical conditions. Other short-term environmental factors could also be important, such as variable feeding behavior or nonsynchronized circadian patterns, to name just a few. However, these are unlikely to explain the correlation between the microbial loads of *H. pylori* and influenza virus because our studies were conducted over the course of about 10 weeks, with *H. pylori* and influenza virus infections separated by at least 8 weeks. Moreover, the experiment was repeated several months later with identical strains and under the same conditions and resulted in a similar outcome. The pathogen bur-

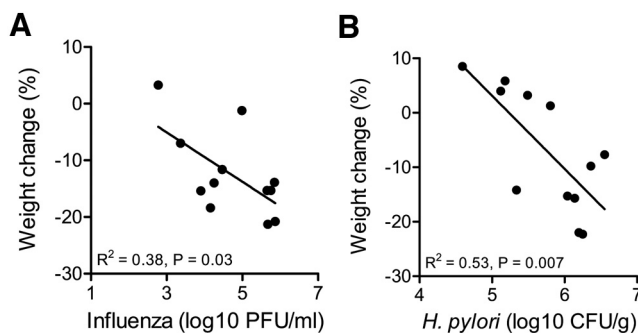


FIG 2 Weight losses due to influenza virus infection correlate with both influenza viral titers and *H. pylori* loads in coinfecting C57BL/6J mice. Age-matched female C57BL/6J mice were inoculated with either *H. pylori* or the carrier and then challenged with influenza A virus 8 weeks later as described in the legend to Fig. 1. Mice were weighed daily from 1 day before to 6 days after an influenza virus challenge, when they were sacrificed. Influenza virus titers and *H. pylori* colonization densities were determined as described in the legend to Fig. 1. The maximal body weight changes (%) correlated significantly with the influenza virus titers in the lungs of mice infected singly with influenza virus (A), as well as the gastric *H. pylori* loads in coinfecting mice (B). Data are pooled from two independent experiments; each symbol indicates a result from an individual mouse.

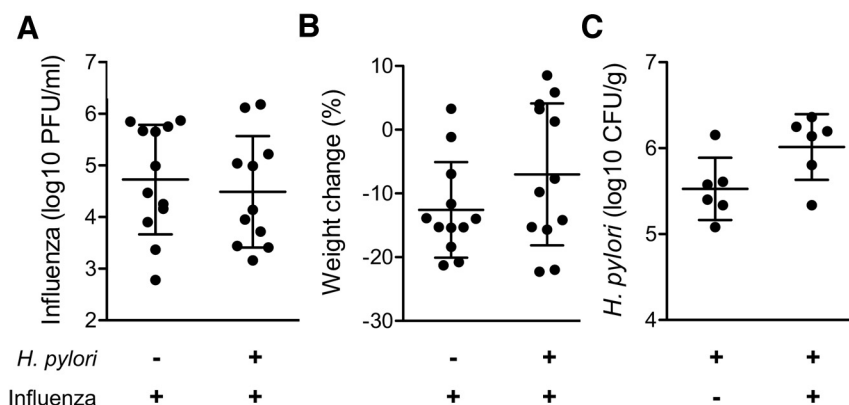


FIG 3 *H. pylori* colonization did not affect the response to influenza virus infection, nor did influenza virus infection affect *H. pylori* colonization. C57BL/6J mice were singly infected with influenza A virus or *H. pylori* or coinfecting with both pathogens as described in the legend to Fig. 1. Neither influenza virus levels (A; $P = 0.62$) nor changes in body weight due to influenza virus infection (B; $P = 0.29$) were affected by *H. pylori*. Data are pooled from two experiments. (C) Similarly, the *H. pylori* load was unaffected by influenza virus infection ($P = 0.09$). Each symbol represents a result from one mouse. Statistical significance values were determined using the two-tailed Mann-Whitney U test; error bars indicate standard deviations from the mean.

den was also not dictated by the overall health of the mice. The growth rate of the mice was not affected by long-term colonization with *H. pylori*, nor was there an association between the weight of the mice prior to influenza virus infection and the influenza virus titer, weight loss, or the *H. pylori* bacterial load (data not shown). Thus, the variability in pathogen burden among inbred mice reflects subtle, individual-specific differences, rather than general fitness.

On the basis of these findings, we are left with the surprising conclusion that inbred C57BL/6 mice, in contrast to common assumptions, are not biologically identical and that the observed phenotypic differences are caused by subtle differences among them. Initially, this seemed a far-fetched possibility, given that these mice have been maintained through inbreeding for over 70 years and it is generally assumed that they are genetically identical. However, the literature provides some evidence that even inbred mice from the same vendor are not isogenic. Gene copy number differences and corresponding differences in gene expression in C57BL/6J mice have been reported (7). A recent transcriptional analysis of individual C57BL/6J mice also noted significant gene expression differences comparing four tissues taken from nonmanipulated mice (8), which was consistent with a previous study (9). Both studies noted expression differences in immune response genes among individual C57BL/6J mice. These differences may affect immunity to viral and bacterial pathogens and could provide a molecular mechanism underlying our observations (Fig. 1).

Gene expression and phenotypic differences among inbred mice may be generated in several ways. For instance, the mouse genome is rife with transposable elements and retroelements alone are responsible for 10% of new mutations among mouse strains (10). While there is strong selection against transposable element variants that impact gene expression, a minority persist (11). New mutations that create single nucleotide polymorphisms (SNPs) or copy number variants could be passed on during inbreeding and result in variable gene expression. Support for the presence of SNPs in C57BL/6J mice was provided independently by two groups that identified differences in the expression of the gene for the insulin-degrading enzyme (7, 8). Thus, it appears that

C57BL/6J mice are heterogeneous with regard to the expression levels of this and potentially other genes. A recent study conducted a full genome analysis of 17 different inbred mouse strains and compared their sequences to the first fully annotated mouse genome deposited in GenBank. The results identified close to 10,000 SNPs between their C57BL/6J sequences and the published one, though some might be due to sequencing errors introduced at a reported rate of about 1/100,000 kb (12). The same study also reported that a considerable part of the mouse genome is not accessible to current sequencing technologies (13% for C57BL/6 mice), leaving open the possibility that there are unrecognized sequence differences among inbred C57BL/6 mice. Together, these data suggest that discrete genetic differences hardwired in the genomes of individual mice may cause differential gene expression, which in turn could affect the outcome of infection.

Mechanisms other than genome sequence differences may also result in differential gene expression. Examples include variation in feeding behavior or epigenetic differences as a result of variable access to nutrients *in utero*, birth order, maternal stress, or other pre- or postpartum events. Such slight differences at birth could be further magnified over time. Differential responses to subtle differences in the local environment may also have an effect on gene expression.

THE HYPOTHESIS: VARIABILITY IN THE RESPONSE TO INFECTION AMONG INBRED MICE REFLECTS BIOLOGICAL DIFFERENCES THAT CAN BE EXPLOITED FOR BIOMARKER DISCOVERY

These observations lead us to draw two important conclusions, one fundamental and the other translational. First, biological differences must exist among individual inbred C57BL/6 mice that affect their response to infectious diseases, despite the fact that they were purchased from the same vendor and housed under nearly identical conditions. Recent studies have demonstrated that inbred mice can have markedly different immune responses to an experimental manipulation, depending upon the composition of their gut microbial community (3, 13). As a result, scientists now pay careful attention to not only the strain, age, and sex of their mice but also the source—even the particular animal

room—from which they derive (14). Now inbred mice are viewed as identical only if they come from the same vendor and are thus colonized with the same or at least very similar microbiota. Here we propose that this assumption, which is the basis of all biomedical research with inbred mice, is wrong. It does not come as a surprise, of course, that not all mice of a given strain behave identically; indeed, scientists anticipate deviation and deal with it statistically by comparing means from sufficiently large groups of animals that the differences between groups are larger than those within groups. But the idea that this variability is, in fact, an inherent property of an individual mouse and not due to experimental error or stochastic effects could fundamentally alter the way in which experiments with inbred mice are conducted, evaluated, and analyzed.

Second, since the *H. pylori* colonization level can predict the influenza viral load and weight loss following a viral challenge, it represents, in effect, a biomarker for predicting the outcome of influenza. While the *H. pylori* colonization level is unlikely to be a clinically useful biomarker, there are, no doubt, other correlates in mice that might predict not only the response to influenza virus but perhaps also the response to other viral or bacterial respiratory pathogens that are clinically relevant. Whether correlations between natural pathogens of the mouse can be observed should also be examined. How could the biological basis of such phenotypic differences be identified? Even sophisticated high-throughput sequencing approaches currently are not powerful enough to unequivocally identify sequencing differences between the genomes of two individual mice. However, determination of phenotypic and transcription level differences may represent a logical and reasonable starting point that would provide the molecular basis for the variability in infection outcomes and could serve as the basis for biomarker discovery to predict the outcome of infectious diseases. Given the expected low rate of genetic differences among individual mice of a given inbred strain, determining the genetic basis of alterations can be readily accomplished through quantitative trait and linkage equilibrium analyses. These discoveries should be much faster than identifying susceptibility-related loci in outbred populations of other animals or, indeed, humans. Thus, the inbred mouse may provide an unprecedented opportunity for the rapid discovery of molecular mechanisms controlling disease outcome and, with it, the potential for identifying predic-

tive biomarkers. Such correlates could provide clinically relevant information for the prevention and treatment of infectious diseases to address some of the immense challenges one faces when having to make rational treatment decisions, especially during resource shortages.

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