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Infection-dependent phenotypes in MHC-congenic mice are not due to MHC: can we trust congenic animals?

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Abstract

Background: Congenic strains of mice are assumed to differ only at a single gene or region of the genome. These mice have great importance in evaluating the function of genes. However, their utility depends on the maintenance of this true congenic nature. Although, accumulating evidence suggests that congenic strains suffer genetic divergence that could compromise interpretation of experimental results, this problem is usually ignored. During coinfection studies with *Salmonella typhimurium* and Theiler's murine encephalomyelitis virus (TMEV) in major histocompatibility complex (MHC)-congenic mice, we conducted the proper F₂ controls and discovered significant differences between these F₂ animals and MHC-genotype-matched P₀ and F₁ animals in weight gain and pathogen load. To systematically evaluate the apparent non-MHC differences in these mice, we infected all three generations (P₀, F₁ and F₂) for 5 MHC genotypes (*b/b*, *b/q* and *q/q* as well as *d/d*, *d/q*, and *q/q*) with *Salmonella* and TMEV.

Results: Infected P₀ MHC *q/q* congenic homozygotes lost significantly more weight ($p = 0.02$) and had significantly higher *Salmonella* ($p < 0.01$) and TMEV ($p = 0.02$) titers than the infected F₂ *q/q* homozygotes. Neither weight nor pathogen load differences were present in sham-infected controls.

Conclusions: These data suggest that these strains differ for genes other than those in the MHC congenic region. The most likely explanation is that deleterious recessive mutations affecting response to infection have accumulated in the more than 40 years that this B10.Q-H-2^q MHC-congenic strain has been separated from its B10-H-2^b parental strain. During typical experiments with congenic strains, the phenotypes of these accumulated mutations will be falsely ascribed to the congenic gene(s). This problem likely affects any strains separated for appreciable time and while usually ignored, can be avoided with the use of F₂ segregants.

Background

It is assumed that knockout, transgenic or other congenic strains of mice are identical at all loci or genomic regions except the one being studied. There are three principal sources of genetic variation that could cause congenic strains to diverge: 1) residual heterozygosity may remain after the construction of the strains, 2) poor animal husbandry could lead to contamination of the strains, and 3) new mutations could become fixed in the strains. There are documented cases in strains of mice of residual heterozygosity [1], genetic contamination [2] and accumulated mutations [3,4]. The relative importance of these three possible sources of genetic divergence among laboratory strains is unclear.

Appreciation of the problem of genetic divergence in strains of inbred mice is not a new one. It has long been expected that independently maintained strains (substrains) might accumulate genetic differences over time [5] and there are now numerous documented cases of phenotypic divergence between substrains. For instance, different substrains of mice respond differently with respect to a stimulus such as cocaine [6] and in cases involving autoimmune or infectious diseases, different substrains of mice respond differently with proteoglycan-induced arthritis [7], experimental allergic orchitis [8], dextran sulfate sodium-induced colitis [9], lipopolysaccharide (LPS) responsiveness [10] and susceptibility to Theiler's virus-induced demyelinating disease [11].

The situation is more problematic for congenic strains because they are expected to be phenotypically different. Thus, when phenotypic differences are detected they are usually attributed to the congenic region without testing (controlling) for the possibility of genetic divergence between strains. The longer congenic mouse strains have been separated, the more likely they will have experienced unintended genetic divergence, particularly from accumulated mutations that can influence detectable phenotypes and thus degrade their congenic nature [12]. The easiest way to test for phenotypic differences that are not due to the known congenic differences is to compare F_2 animals to their genotype matched P_0 and F_1 counterparts. If the only differences are the congenic genes, then the F_2 results will match those from P_0 and F_1 animals. If other unknown differences, such as accumulated mutations, exist between these congenic strains, then F_2 results will differ from the P_0 and F_1 results because the unknown genetic differences will be randomly segregating in the F_2 animals. Unfortunately, the problem is usually ignored and if present will be undetected without conducting F_2 controls. Without these controls, the phenotypes due to accumulated mutations will be falsely ascribed to the congenic region.

During coinfection studies designed to evaluate the role of MHC genotypes on infection with *Salmonella* and a virus that induces symptoms similar to multiple sclerosis, Theiler's murine encephalomyelitis virus (TMEV) [13], we conducted the F_2 controls to determine if these animals behaved differently from P_0 and F_1 animals with respect to weight gain and pathogen load. We suspected they might, because the B10 MHC congenic strains used have been separated for greater than 40 years, enough time for many mutations to become fixed in these strains. Since we observed significant differences between the P_0 , F_1 and F_2 generations of mice, we completed that study with F_2 animals to avoid the problem of accumulated mutations in these lines [13].

To experimentally explore this apparent mutation accumulation in the B10 MHC-congenic lines, we conducted an additional experiment (reported here) where sham-infected and infected P_0 , F_1 and F_2 mice were assayed for differences in weight gain and pathogen titer in this coinfection model. We report significant infection-dependent differences between generations. This study is the first time this problem has been documented in B10 MHC-congenic strains and suggests that deleterious recessive mutations or other sources of genetic divergence have destroyed the purported congenic nature of these strains.

Results

Table 1 provides the *Salmonella* and TMEV titers for the infected mice. The F_2 q/q homozygotes had significantly lower *Salmonella* titers than the P_0 q/q homozygotes for the b and q genotypic combination ($F(1,35) = 7.13$, $p = 0.01$) and a marginally significant trend in the d and q combination ($F(1,33) = 3.02$, $p = 0.09$). Similarly, the F_2 q/q homozygotes had significantly lower TMEV titers than the P_0 q/q homozygotes for the d and q genotypic combination ($F(1,33) = 5.85$, $p = 0.02$). The pattern was similar for the b and q genotypic combination but the difference was not significant.

Figure 1 illustrates the weight change for both the sham-infected and infected d and q genotypic combination of mice. All infected mice had retarded growth as compared with the sham-infected mice (Figure 1, $F(1,188) = 37.839$, $p < 0.0001$). The F_2 q/q homozygotes lost significantly less weight than the P_0 q/q homozygotes (Figure 1, $F(1,165) = 5.79$, $p = 0.02$) and this was consistent with the *Salmonella* and TMEV titers (Table 1). The weight-difference pattern was similar between P_0 and F_2 q/q mice in the b and q genotypic combination (data not shown), but it was not significant. There was no significant difference between P_0 and F_2 q/q mice that were sham-infected.

Table 1: Average *Salmonella* and TMEV titers (+/- SE) for female P₀, F₁ and F₂ mice.

Generation	Sample Size	MHC Genotype	<i>Salmonella</i> titers (10 ² CFU/spleen)	TMEV titers (10 ² PFU/spcd)
P ₀	6	b/b	51.93 +/- 6.60	1.33 +/- 1.09
F ₁	8	b/q	6.60 +/- 0.83	0.97 +/- 0.41
P ₀	7	q/q	13.09 +/- 3.27^a	126.75 +/- 32.75
F ₂	7	b/b	140.49 +/- 38.87	4.00 +/- 2.63
F ₂	7	b/q	6.34 +/- 1.58	3.21 +/- 1.99
F ₂	6	q/q	5.67 +/- 2.00^a	52.08 +/- 16.63
P ₀	7	d/d	10.57 +/- 1.21	4.29 +/- 3.55
F ₁	7	d/q	7.23 +/- 1.80	11.25 +/- 6.04
P ₀	6	q/q	10.53 +/- 3.60^c	331.92 +/- 180.16^b
F ₂	4	d/d	9.05 +/- 1.19	3.81 +/- 3.40
F ₂	10	d/q	14.00 +/- 4.11	6.15 +/- 1.76
F ₂	5	q/q	4.16 +/- 0.85^c	13.65 +/- 8.98^b

spcd = spinal cord; Comparisons in bold are significantly different as follows: ^a (F(1,35) = 7.127, p = 0.01); ^b (F(1,33) = 5.84, p = 0.02); ^c marginally significant (F(1,33) = 3.02, p = 0.09).

Discussion

In four of six comparisons, F₂ q/q homozygotes did significantly better during coinfection than their P₀ counterparts as measured by pathogen titers (Table 1) or weight change (Figure 1). These results are consistent with the hypothesis that deleterious recessive mutations influencing susceptibility to *Salmonella* and/or TMEV infection have accumulated in this B10.Q MHC congenic strain. This conclusion is not too surprising, given that these MHC-congenic strains have been separated for more than 40 years and recent experiments [14] and theory [15] have shown that important mutations can accumulate in a small number of generations. In addition, the effective population size of the breeding colonies at The Jackson Laboratory is usually about 24 individuals (12 foundation breeding pairs, Jennifer Merriam, The Jackson Laboratory, personal communication), causing drift to be a powerful factor in the fixation of deleterious mutations [16]. This is the first study to identify infection-dependent phenotypes in MHC-congenic strains that are not due to genes within the MHC congenic region. Many studies have identified infection-dependent phenotypes between MHC-congenic strains ([13], and references therein), but none of those studies tested whether the phenotype segregates with the congenic region by testing F₂ segregants.

Since F₂ q/q mice lose less weight (Figure 1) and show a decrease in pathogen load relative to P₀ q/q mice (Table 1), these data suggest that the observed genetic divergence is due to the accumulation of deleterious recessive mutations, rather than genetic contamination or residual heterozygosity. We make this interpretation because among the three possible sources of genetic divergence, only random mutations are usually expected to be deleterious recessive. Predictions can be made about how accumulated recessive

deleterious mutations will be expressed in each generation. Homozygous P₀ will express all deleterious recessive mutations while the F₁ heterozygotes will experience hybrid vigor (or heterosis) because the inherited recessive deleterious mutations will not be in a homozygous state and the defective phenotypes will be masked. In F₂ segregants, the inheritance of any recessive mutations will be randomized such that some mutations will be expressed (in a homozygous state) and some mutations will not be expressed (in a heterozygous state). Thus, if recessive deleterious mutations are present, the F₂ segregants should show an increase in weight gain and a decrease in pathogen titers compared to the P₀ mice, as was generally observed in this study. The F₂ segregants might have a higher variance, but because the expression of deleterious recessive mutations is randomized, it will still be possible to evaluate the phenotype of the congenic gene(s). Thus, using congenic F₂ segregants should eliminate any bias caused by accumulated mutations [17], except those linked to the congenic region.

The fixation of deleterious mutations causing infection-dependent susceptibilities (as we observed in weight change, Figure 1), would be expected to occur disproportionately in animal strains housed in "clean" (pathogen free) colonies. Mutational defects that are infection-dependent should accumulate in pathogen free colonies because they carry little to no cost in the absence of infections. Because most, if not all, inbred mice have been housed in pathogen free colonies for numerous years, this predicts that infection-dependent differences between strains might accumulate at higher rates than other mutational defects.

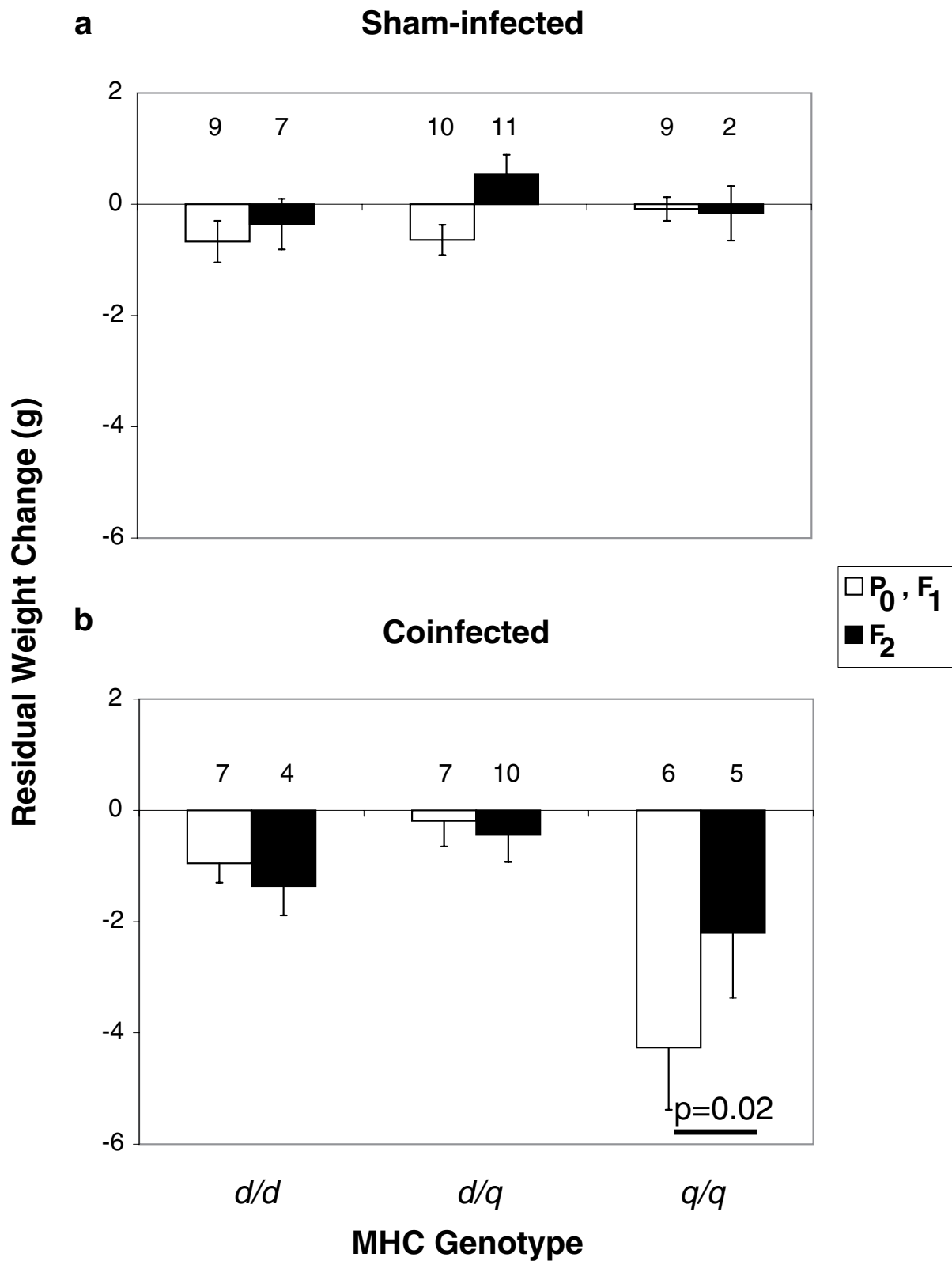


Figure 1
 Residual weight change (+/- SE) between sham-infected (a) and coinfecting (b) P₀, F₁ and F₂ female mice. Sample sizes are indicated above each bar. All p values are from an ANOVA with simple comparisons.

The ability to detect mutation accumulation is easier in substrains that are purportedly identical than among congenic strains. This is because substrains are expected to express identical phenotypes and when differences are detected they are usually interpreted as due to mutational divergence. In contrast, when differences are detected among congenic strains, the typical interpretation is that the congenic region caused these differences. Unfortunately, the differences could be due to accumulated mutations [3,4], residual heterozygosity [1] or genetic contamination [2] of the congenic strain. There is no way to discriminate between phenotypes due to the congenic region from those due to genetic divergence without additional experiments, such as conducting F_2 controls, but these additional experiments are almost never done. One must be suspicious of the 33 phenotypes that have been ascribed to MHC genes [18], many of which have nothing to do with immune recognition. We suggest that many of these phenotypes are due to accumulated mutations or genetic divergence.

Because MHC congenic strains are among the oldest of congenic strains, they have been tested for many phenotypes over the years. One recent example involves the claim that an "electronic nose" could detect odor differences between MHC mutational congenic strain bm1 and the parental B6 that differs by only 3 mutations in a class I binding site [19]. This study did not use F_2 controls. A subsequent study using an odor detection assay with mice confirmed that these two congenic strains did have odor differences detectable by mice, but these differences disappeared when F_2 animals were tested [20]. This olfaction study further supports the conclusions of this paper that these B10 congenic strains have diverged genetically causing phenotypic differences not due to the MHC congenic region.

Conclusions

Any strain separated by appreciable amounts of time will invariably accumulate mutational differences and this study demonstrates that these accumulated mutational differences can be important. For congenic strains, these mutational differences will be falsely ascribed to the congenic region when comparing pure strains. While we only have evidence for mutation accumulation in the q/q congenic strains, it is unlikely that other congenic strains have not accumulated mutations. This problem is widely ignored. Re-deriving the strains frequently or using F_2 segregants can solve this problem. Unfortunately, both remedies are time-consuming and expensive, particularly re-deriving the strains.

Methods

Mice

MHC congenic mice (C57BL/10SnJ- $H2^b$, B10.D2- $H2^d$ $H2-T18^c$ $Hc^1/nSnJ$ and B10.Q- $H2^q$ /SgJ) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred thereafter under specific pathogen free conditions. P_0 homozygotes were bred to produce F_1 heterozygote offspring. These F_1 mice were then intercrossed to produce the F_2 segregants. P_0 , F_1 and F_2 mice were either sham-infected with phosphate buffered saline (PBS) or infected with *Salmonella enterica* (serovar Typhimurium, strain C5TS) and the Daniels' (DA) strain of TMEV.

To control for potential heterosis, only F_2 segregants derived from the F_1 heterozygotes were used in the experiment. To control for potential variation among cages, age-matched mice were housed by sex, in groups of six individuals representing the three genotypes to be compared (e.g. b/b , b/q and q/q or d/d , d/q and q/q) from both the F_2 and P_0 or F_1 generation. All q/q mice were analyzed according to their housing conditions and thus not pooled for analysis (e.g. q/q mice housed with b/b and b/q mice were analyzed only with the b and q haplotypic combination). All cagemates were unrelated (progeny of different parents) and had not been previously housed together when infected. Mice were MHC-genotyped at two microsatellite loci within the MHC region (a tetranucleotide repeat [21] and d17Mit34 [22]) using PCR for DNA amplification and denaturing gel electrophoresis for scoring band size.

Weights

Mice infected with *Salmonella* or TMEV display noticeable weight loss that is usually correlated with disease symptoms. Thus, mice were weighed three times per week. The following regression equations were based on sham-infected weights and take into account the starting weight and age of the mice. These equations were used to calculate the residual weight change in order to control for 1–2 week differences in the starting ages of the mice. For female mice, residual weight change = $34.3065 - 1.5151(\text{Age}) + 0.0221(\text{Age})^2 - 0.00009(\text{Age})^3$.

Parameters of infection

Four-to seven-week-old mice were anesthetized with metofane (methoxyflurane) and simultaneously infected retro-orbitally with 1×10^6 CFU/ml of the C5TS strain of *Salmonella* and intra-cerebrally with 2×10^5 PFU/ml of the DA strain of Theiler's virus or sham-infected as above with PBS. The C5TS strain is a temperature-sensitive mutant strain of *Salmonella* [23] that causes a chronic, non-fatal infection. The overnight culture of *Salmonella* was washed twice with PBS to remove any excess LPS on the surface of the bacteria before mice were infected.

Salmonella titers

Mice were sacrificed four weeks post-infection. *Salmonella* titers were assayed from platings of homogenized spleen. Briefly, spleens were collected under antiseptic conditions, immediately homogenized and plated on *Shigella-Salmonella* (SS) agar, which was then incubated overnight at 30°C. When this strain was created in the early 1970s [23] with an UV screen, the authors found that a small (unspecified) percentage reverted back to the wild-type C5 virulent strain. To check for possible wild-type revertants, spleen homogenates were also plated on SS agar and incubated overnight at 37°C. If more than 50% of the colonies of an infected mouse also grew at 37°C, then the infection was deemed to be dominated by a revertant and the data point was excluded on the basis that the revertant was more lethal (i.e. faster replication rate than C5TS). Only one animal contained <50% revertants. A total of 58 animals (out of 228) were excluded either because they died, were sacrificed prior to 28 days or contained *Salmonella* revertants. Of the 58 excluded mice, 13 (22%) died, 33 (57%) were sacrificed prior to 28 days due to severe illness and 12 (21%) contained revertants. Of the 33 mice that were sacrificed early, 21 (63%) contained revertants.

Theiler's virus titers

TMEV titers were assayed by conducting viral plaque assays on brain and spinal cord homogenates [24]. Briefly, brain and spinal cords were collected and stored in 0.5 ml TMEV diluent (PBS, 1% antibiotics, 1% fetal calf serum) at -70°C until assayed. Brain and spinal cords were homogenized and frozen/thawed twice. Samples were then centrifuged at 450 g for 10 minutes and the supernate collected. Supernatant fluid was then added to baby hamster kidney (BHK) -21 cells and incubated for one hour at 37°C, hand rocking every 10–15 minutes. Fluid was then aspirated off the BHK cells and a 1:1 mixture of 1% agarose: 2X 199 Medium was added to the wells. Plates were incubated at 37°C for 4 days before fixation with 2.5% formalin, followed by staining with 0.1% crystal violet and enumeration of plaques.

Statistics

An analysis of variance (ANOVA) was used to test for weight differences between MHC genotype and generation (P_0 , F_1 versus F_2) while a multivariate analysis of variance (MANOVA) was used to test for pathogen load differences between MHC genotype and generation (P_0 , F_1 versus F_2). Because an increase in type I error is controlled for by use of a MANOVA, this was followed by univariate analyses for each pathogen for each genotypic combination. Where needed, simple contrasts were then done to determine which genotype(s) was responsible for the effect.

Abbreviations

P_0 , parental MHC-congenic mice from The Jackson Laboratory; F_1 , MHC-heterozygote offspring of two parental MHC-congenic strains; F_2 , F_2 segregants resulting from a F_1 MHC-heterozygote \times F_1 MHC-heterozygote cross.

Authors' contributions

EEM helped design the experiment, set up mouse breeding cages and helped wean mice, infected and sacrificed all mice, calculated pathogen titers, performed data and statistical analysis and drafted the manuscript. KD and ZJG weighed sham-infected and infected mice. KG, KSR and MW helped wean, genotype, weigh, infect and sacrifice mice. MSM and MN did data entry. LCM did data entry and trained undergraduates in molecular genetic techniques. PB performed statistical analysis. WKP participated in the design and data analysis of the study and helped draft the manuscript.

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