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

Erin E McClelland*1, Kristy Damjanovich1, Kyle Gardner1, Zack J Groesbeck1, Maggie S Ma1, Megan Nibley1, Kelly S Richardson1, Maureen Wilkinson1, Linda C Morrison1, Paul Bernhardt2 and Wayne K Potts1

Address: 1Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112 USA and 2Department of Educational Psychology, University of Utah, 1705 East Central Campus Drive, Salt Lake City, UT 84112 USA

Email: Erin E McClelland* - mcclella@aecom.yu.edu; Kristy Damjanovich - kdamjanovich@cc.utah.edu; Kyle Gardner - kylegard@yahoo.com; Zack J Groesbeck - zman_groesbeck@hotmail.com; Maggie S Ma - maggie_s_ma@yahoo.com; Megan Nibley - megan.nibley@m.cc.utah.edu; Kelly S Richardson - ks.richardson@m.cc.utah.edu; Maureen Wilkinson - tiger_lily_blue@yahoo.com; Linda C Morrison - lmorrison@biology.utah.edu; Paul Bernhardt - Paul.Bernhardt@ed.utah.edu; Wayne K Potts - potts@biology.utah.edu

* Corresponding author

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-2q MHC-congenic strain has been separated from its B10-H-2b 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 F2 animals to their genotype matched P0 and F1 counterparts. If the only differences are the congenic genes, then the F2 results will match those from P0 and F1 animals. If other unknown differences, such as accumulated mutations, exist between these congenic strains, then F2 results will differ from the P0 and F1 results because the unknown genetic differences will be randomly segregating in the F2 animals. Unfortunately, the problem is usually ignored and if present will be undetected without conducting F2 controls. Without these controls, the phenotypes due to accumulated mutations will be falsely ascribed to the congenic region.

During co-infection 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 F2 controls to determine if these animals behaved differently from P0 and F1 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 P0, F1 and F2 generations of mice, we completed that study with F2 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 P0, F1 and F2 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 F2 q/q homozygotes had significantly lower Salmonella titers than the P0 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 F1 q/q homozygotes had significantly lower TMEV titers than the P0 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 F2 q/q homozygotes lost significantly less weight than the P0 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 P0 and F2 q/q mice in the b and q genotypic combination (data not shown), but it was not significant. There was no significant difference between P0 and F2 q/q mice that were sham-infected.
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.

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

| Generation | Sample Size | MHC Genotype | Salmonella titers (10^2 CFU/spleen) | TMEV titers (10^2 PFU/spcd) |
|------------|-------------|--------------|------------------------------------|-----------------------------|
| P0         | 6           | b/b          | 51.93 +/- 6.60                     | 1.33 +/- 1.09               |
| F1         | 8           | b/q          | 6.60 +/- 0.83                      | 0.97 +/- 0.41               |
| P0         | 7           | q/q          | 13.09 +/- 3.27*                    | 126.75 +/- 32.75            |
| F2         | 7           | b/b          | 14.049 +/- 38.87                   | 10.57 +/- 1.21              |
| F2         | 7           | b/q          | 6.34 +/- 1.58                      | 3.21 +/- 1.99               |
| F2         | 6           | q/q          | 5.67 +/- 2.00a                     | 52.08 +/- 16.63             |
| P0         | 7           | d/d          | 10.57 +/- 1.21                     | 4.29 +/- 3.55               |
| F1         | 7           | d/q          | 7.23 +/- 1.80                      | 11.75 +/- 6.04              |
| P0         | 6           | q/q          | 10.53 +/- 3.60a                    | 33.92 +/- 180.16b           |
| F2         | 4           | d/d          | 9.05 +/- 1.19                      | 3.81 +/- 3.40               |
| F2         | 10          | d/q          | 14.00 +/- 4.11                     | 6.15 +/- 1.76               |
| F2         | 5           | q/q          | 4.16 +/- 0.85*                     | 13.65 +/- 8.98b             |

spcd = spinal cord. Comparisons in bold are significantly different as follows: * (F(1,35) = 7.127, p = 0.01); ** (F(1,33) = 5.84, p = 0.02); <marginally significant (F(1,33) = 3.02, p = 0.09).
Residual weight change (+/- SE) between sham-infected (a) and coinfected (b) P0, F1 and F2 female mice. Sample sizes are indicated above each bar. All p values are from an ANOVA with simple comparisons.

Figure 1
Residual weight change (+/- SE) between sham-infected (a) and coinfected (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 con- genic 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 F2 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 F2 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 F1 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 F2 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-Hc\(1\)/nSnJ and B10.Q-H2\(d\)/(SgJ) were obtained from The Jackson Laboratory (Harbor, ME) and bred thereafter under specific pathogen free conditions. P0 homozygotes were bred to produce F1 heterozygote offspring. These F1 mice were then intercrossed to produce the F2 segregants. P0, F1 and F2 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 F2 segregants derived from the F1 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 F2 and P0 or F1 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(Age) + 0.0221(Age)\(2\) – 0.00009(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\(^3\) 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 lablings 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 (P0, F1 versus F2) while a multivariate analysis of variance (MANOVA) was used to test for pathogen load differences between MHC genotype and generation (P0, F1 versus F2). 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.

Salmonella

**Abbreviations**

F0, parental MHC-congenic mice from The Jackson Laboratory; F1, MHC-heterozygote offspring of two parental MHC-congenic strains; F2, F2 segregants resulting from a F1 MHC-heterozygote × F1 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.

**Acknowledgements**

We thank Jane Libbey and Elena Enioutina for excellent technical assistance; Robert Fujinami and Janis Weis for helpful comments and Carlos Horeanche for donation of the Salmonella strain C5TS. This work was supported by an NIH grant to W.K.P. (GM39578) and partially by an NIH grant to Robert S. Fujinami (NS34497). All animal use complied with United States federal regulations and the University of Utah's Institutional Animal Care and Use Committee guidelines.

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