Seroconversion of 1-year-old Mice to Murine Norovirus

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Rodent sentinel screening for adventitious pathogens is an integral part of many biomedical research institutes and universities that use rodents in research. Typical screening programs involving live sentinel animals typically purchase young SPF sentinel animals that are sampled and replaced quarterly. Previous reports suggest that mice as old as 6 mo are effective sentinels for various agents. In efforts to reduce the number of animals used in our sentinel program, we wanted to investigate the possibility of keeping sentinel animals inhouse for 12 mo at a time. We exposed mice (age, 40 to 48 wk) to murine norovirus (MNV) to test whether they could reliably produce detectable levels of antibodies (similar to younger mice) to this adventitious pathogen. Mice first exposed to MNV at 40 to 48 wk of age seroconverted to MNV after both direct inoculation (through gavage) and indirect exposure (from soiled-bedding transfer) at the same or greater frequency than mice first exposed at 8 to 12 wk of age. These findings indicate that, at least for MNV, sentinel residence time can be extended from 3 to 12 mo without compromising the reliability of seroconversion, thus ultimately reducing sentinel animal numbers. This practice, combined with nonanimal testing modalities (for example, exhaust duct sampling), can increase the sensitivity and specificity of rodent surveillance programs and minimize the use of live animals.

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Rodent sentinel screening for adventitious pathogens is an integral part of the animal care programs of many biomedical research institutes and universities that use rodents in research. Review of sentinel health monitoring protocols for many biomedical research institutions reveals a general age-related preference to use young (4- to 10-wk-old) sentinel animals.⁶ Typical screening programs involving live sentinel animals typically purchase young sentinel mice and rats quarterly and use soiled-bedding transfer or direct exposure methods (or both) to sample colony animals. In large institutions, this practice can amount to hundreds to thousands of animals used every year. Compared with their older counterparts, young mice are thought to be more susceptible to various infectious agents (for example, mouse parvovirus) and can mount robust serologic responses.² Studies have shown that old mice (that is, 18 mo or older) have reduced immunologic responsiveness.^{5,9,10} In addition, facilities may have different priorities and needs regarding sentinel health screening of rodent colonies, including different pathogens that are excluded or allowed to be endemic in populations. For example, *Helicobacter* is not routinely excluded from all facilities. However, for some studies that would be adversely affected by *Helicobacter* in the colony, facilities may choose a sentinel strain that will be more sensitive and permit earlier detection of *Helicobacter* infection.

In efforts to reduce the numbers of rodents used in our laboratory animal sentinel program, we wanted to investigate the possibility of keeping sentinel animals inhouse for periods of 12 mo compared with 3 mo. Therefore, we needed to directly test whether sentinel mice first exposed to a typical murine

adventitious pathogen, murine norovirus (MNV), at 10 to 12 mo of age could reliably produce detectable levels of antibodies. MNV is a ubiquitous viral pathogen in mice used for biomedical research, and exposure to MNV through soiled-bedding transfer has been validated.^{7,8} MNV is primarily a subclinical but persistent infection, and immunocompetent animals with MNV are asymptomatic with prolonged fecal shedding.³ However, some immunocompromised mice infected with MNV will show clinical signs including weight loss and hunched posture and pathologic changes in the gastrointestinal tract, liver, and immune system.¹¹ Therefore, continued screening for and eradication of MNV from rodent colonies is prudent given that infection with MNV may modify the immune system and can interfere with studies involving the enteric system.⁴ Finally, using MNV transmission through soiled-bedding transfer in the current study provides additional important data regarding this agent, which has been difficult to detect in this manner.^{2,12}

In this study, we compared the ability to detect antibodies to MNV by using standard serologic methods of mice first exposed to MNV4 at 40 to 48 wk of age (that is, older mice) compared with those exposed at 8 wk of age (that is, young mice). Mice were inoculated directly through gavage of MNV4 or were exposed indirectly through weekly soiled-bedding transfer. The results indicated that mice first exposed at 40 to 48 wk of age are just as able to produce detectable antibodies as are mice exposed at 8 wk of age, by both direct and soiled bedding exposure routes. These findings contribute to the application of 3Rs principles and represent a viable mechanism to reduce the number of animals used when moving to nonanimal sentinel methodology is not possible.

Materials and Methods

MNV. Frozen cultures of MNV4 (2×10^8 pfu/ μ L) were generated as previously described.^{3,7} The MNV4 vials were kept frozen at -80°C until use, when they were diluted with ice-cold

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PBS (Sigma, St Louis, MO) to a final concentration of 1×10^6 pfu in 200 μ L for each animal gavaged (in the dirty-bedding donor and direct inoculation groups).

Mice. The C57BL/6J (hereafter C57BL/6) and BALB/cJ (hereafter BALB/c) mice used in this study came from Jackson Labs (Bar Harbor, ME). All mice were documented to be free of ectoparasites, endoparasites, *Mycoplasma pulmonis*, *Helicobacter* spp., known enteric and respiratory bacterial pathogens, and antibodies to mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler murine encephalomyelitis virus, *Ectromelia* virus, polyoma virus, lymphocytic choriomeningitis virus, mouse adenovirus, minute virus of mice, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus mouse thymic virus, murine norovirus, *Encephalitozoon cuniculi*, and *Clostridium piliforme*.

All mice were housed in the same animal room in individually ventilated racks (Allentown Caging, Allentown, New Jersey) in cages autoclaved with aspen chip bedding (Teklad, Envigo, Indianapolis, IN) and various nesting materials, including autoclaved cotton squares (Ancare, Bellmore, NY) or autoclaved cardboard tubes (toilet paper rolls) for enrichment. Irradiated rodent chow (Teklad, Envigo) and autoclaved water were provided without restriction. Complete cage changes were done every 2 wk; food and water were topped off and extra enrichment added as needed weekly.

The animal holding room was maintained at 70 to 72 °F (21.1 to 22.2 °C), at 30% to 70% humidity, on a 12:12-h light:dark cycle, and at 15 fresh-air changes hourly. All handling and manipulations of mice were conducted under a vertical laminar flow animal transfer station (NuAire, Plymouth, MN). Between cage changes, the station was wiped with chlorine dioxide solution (MB10, Quip Labs, Wilmington, DE).

The University of Miami Animal Care and Use Committee approved all experiments. MNV infection by inoculation or soiled-bedding exposure was performed in young mice (8 wk of age at start) and older mice (40 to 48 wk of age at start). The study included 2 experimental groups: those directly inoculated with MNV (by gavage), and those exposed by using soiled-bedding transfer. An additional 15 C57BL/6 mice (female; age, 8 wk of age; $n = 3$ cages) were used as the soiled-bedding donors for the soiled-bedding exposure experiment.

MNV antibodies were detected from blood samples. Blood samples (1 or 2 drops each) were collected from the submandibular vein and placed onto an Opti-Spot card (Idexx Bioresearch, Columbia, MO). The sample-bearing cards were sent to Idexx laboratories for multiplex fluorescent immunoassay serologic analyses according to inhouse protocols. For MNV RT-PCR analysis, 3 to 5 fecal pellets were collected from each cage and sent to Idexx for analysis.

Direct inoculation study. To verify whether older mice seroconvert to MNV, 13 older mice (9 C57BL/6 and 4 BALB/c; age [mean $\pm$ 1 SD], 44.9 ± 2.2 wk) and 16 young mice (8 C57BL/6 and 8 BALB/c female; age, 8 wk of age) were inoculated (via gavage) with 1×10^6 pfu MNV4 in 200 μ L PBS. Mice were group housed (2 to 3 mice/cage) throughout the study. RT-PCR for MNV on feces per cage verified that all mice were MNV negative at study start. Beginning at 8 wk postgavage, a sample of blood for MNV serology was taken from each mouse and a sample of fresh feces (collected within 48 h after cage change) for MNV RT-PCR was collected from each cage every 2 wk until 12 wk. At 12 wk postgavage, all animals were euthanized by carbon dioxide intoxication and cervical dislocation, and a terminal blood sample and cage fecal sample were collected.

Soiled bedding exposure study. MNV4-positive soiled bedding was generated by inoculating 15 female C57BL/6J mice (age, 8 wk; housed 5 per cage in 3 cages) with 1×10^6 pfu MNV4 in 200 μ L PBS by gavage. Before inoculation, these mice were confirmed to be MNV negative (by individual serology and cage-level fecal RT-PCR assay for MNV). At 2 wk after gavage, fresh feces (collected within 48 h after cage change) were obtained for MNV RT-PCR to confirm MNV exposure and excretion. Every 2 wk thereafter, fecal samples from each of the 3 soiled-bedding donor cages were analyzed for MNV by RT-PCR assay.

We wished to verify whether older mice reliably mounted antibody responses through weekly exposure to MNV-positive soiled bedding. Older C57BL/6J ($n = 10$ [4 cages]; age, 48 wk), BALB/c ($n = 8$ [4 cages]; age, 44.3 ± 2.7 wk at study start), and 8 each of young BALB/c and C57BL/6 female (age, 8 wk; pair-housed; $n = 8$ cages) each received 15 cm³ of MNV-positive soiled bedding from soiled-bedding donor cages. The volume of 15 cm³ of soiled MNV-positive bedding was chosen in light of previous MNV transmission studies indicating 80% positive transmission in animals at 2 wk after gavage of this volume.⁷ Weekly before each soiled-bedding transfer, bedding from the 3 donor cages was combined, and 15 g (equivalent to 15 cm³) was collected and transferred to each cage in the soiled-bedding exposure groups. Weekly, fresh MNV-positive soiled bedding was added, and every 2 wk, half of the dirty bedding was removed and placed into a clean cage; fresh clean bedding added as well. Beginning at 8 wk after the first soiled-bedding transfer, a sample of blood for MNV serology was taken from each mouse and a sample of feces for MNV RT-PCR analysis was collected from each cage every 2 wk until 12 wk after the first soiled-bedding transfer. At 12 wk, all mice were euthanized by CO₂ intoxication and cervical dislocation, and a terminal blood sample and cage fecal sample were collected.

Statistical analyses. Fisher exact tests (Prism 7.01, GraphPad Software, La Jolla, CA) were used to compare numbers of animals that seroconverted to MNV and that were positive by RT-PCR analysis for MNV between older and young animals. Significance was set at a P value of 0.05.

Results

Beginning 8 wk after direct inoculation with MNV4 (and through 12 wk after inoculation), all 13 older and 16 young mice had detectable antibodies to MNV (Figure 1 A). In addition, mice in all cages were positive for MNV by fecal RT-PCR analysis at 8 wk. However, no clinical signs of infection were evident in any of these mice.

At 8 wk after indirect exposure to MNV through virus-positive soiled bedding, serologic conversion to MNV was detected in 2 of the 8 young C57BL/6 animals and in 6 of the 8 BALB/c mice (Fisher exact test, $P = 0.13$; Table 1). Because these values were not statistically different, they were combined for analysis. Overall at 8 wk after exposure, more (Fisher exact test, $P = 0.03$) older animals had detectable antibodies to MNV than did young animals (Figure 1 B) despite approximately equal numbers of RT-PCR-positive cages (young mice, 6 of 8 cages; older mice, 7 of 8 cages; $P > 0.05$).

By 10 wk after exposure, the remaining individual mice had detectable antibodies, and all remaining cages were positive for MNV by fecal RT-PCR analysis. Ultimately, by 12 wk after exposure, one cage of young C57BL/6 mice had never produced MNV-positive feces.

Soiled-bedding donor cages were sampled every 2 wk to confirm MNV positivity through RT-PCR analysis. Beginning at 2 wk after gavage and throughout the course of the study,

Figure 1. (A) Percentages of mice with antibodies to MNV at 8, 10, and 12 wk after direct exposure to MNV (gavage) for young (age at exposure, 8 wk) and older (age at exposure, 40 to 48 wk) C57BL/6J and BALB/c mice. (B) Percentages of seropositive mice at 8, 10, and 12 wk after indirect exposure to MNV (weekly soiled-bedding transfer) for young (age at exposure, 8 wk) and older (age at exposure, 38 to 40 wk) C57BL/6J and BALB/c mice. *, Value significantly ($P < 0.05$) different between older and young mice at the 8-wk time point.

Table 1. MNV shedding from and seroconversion of young and older C57BL/6 and BALB/c mice at 8 wk after inoculation or exposure to MNV-positive soiled bedding

	No. of cages positive for MNV ($n = 4$ analyzed)	No. of individual mice positive for MNV ($n = 8$ analyzed)
Young C57BL/6	3	2
Young BALB/c	3	6
Older C57BL/6	4	8
Older BALB/c	3	6

MNV shedding was determined by fecal RT-PCR analysis.

all donor cages were positive for MNV by RT-PCR assay. There were no clinical signs or evidence of disease in any animal in any of the treatment groups.

Discussion

This study demonstrates that mice first exposed to MNV at 40 to 48 wk of age reliably seroconvert and produce antibodies at levels similar to or greater than those produced by mice that are 8 wk old at first exposure. A few studies have examined the seroconversion efficiency of mice as old as 6 mo at first exposure or when mice are very old (for example, 16 mo or older).^{1,4,9} In one study,² Swiss Webster sentinel mice that were 4, 12, or 44 wk old were equally effective in seroconverting to mouse parvovirus and mouse hepatitis virus after exposure by soiled-bedding transfer. In addition, among all age groups, the 44-wk-old sentinels were the only ones that successfully transmitted MNV, albeit transmission occurred in only 25% of these mice. Furthermore, MNV transfer in soiled-bedding

sentinels is not always efficient, and MNV can be shed at lower titers compared with other viruses.¹² Therefore, the efficient transmission and seroconversion demonstrated in our current study is impressive and further suggests that older mice may be very useful as sentinels.

Typical recommendations for rodent sentinel health programs include the use of young sentinels (for example, 4 to 10 wk of age) to optimize seroconversion and antibody production.⁶ Immunologic response decreases with age in both humans and mice.^{5,9,10} However, many of the cited studies focused on specific B- or T-cell markers or cytokines, and many involved in vitro or ex vivo experiments. Although informative, these previous studies do not necessarily indicate the seroconversion efficiency to mildly pathogenic agents typically screened in laboratory rodent colonies. Nor do the findings indicate the ability to seroconvert to repeated exposure to low doses of pathogen through soiled-bedding transfer. Therefore, we wanted to directly examine seroconversion in older mice (that is, approximately 12 mo of age at exposure).

Currently our sentinel program for mice follows commonly recommended practices: using young mice (8 to 10 wk of age), using outbred (CD1) or inbred strains known to have robust immunologic responses (for example, BALB/c), and replacing sentinels quarterly after screening. We use a single sentinel mouse (CD1) per side of double-sided mouse rack (which thus receives samples from 40 to 70 cages, depending on rack type) in a soiled-bedding transfer system of exposure. The recent advances in environmental and colony sampling using exhaust duct sampling (no animals used) and established RT-PCR methodologies support modifications of the traditional surveillance practices. For example, sentinels in residence for 12 mo can be survival-bled quarterly until the end of the 4th quarter, when they would be euthanized for full necropsy. In addition, nonterminal bleeding of long-term sentinels can be interspersed with environmental sampling using RT-PCR methodologies to reduce the total number of blood collections (that is, refinement).

In particular, we were interested in decreasing the number of live sentinels used overall. However, before switching to a new practice, we wanted confirmation that mice residing in facility for 12 mo would still produce a reliable antibody response that was detectable by common methodology. We chose MNV as the test pathogen, because it is commonly screened and is endemic in our facilities, thereby offering little to no risk.

At the time of study inception, geriatric C57BL/6 and BALB/c mice were available from a previous study examining fecal microbiome changes due to various diets. No experimental procedures were performed on these mice, other than fecal collection. Although not typically used as sentinels, C57BL/6 mice are the most common strain in our facility, and we wanted to investigate their potential use as sentinels. Given that outbred CD1 and BALB/c mice are considered to be more robust seroconverters, the fact that we found high levels of seroconversion in older C57BL/6 mice suggests that this strain may in fact be viable for use as sentinels, at least for MNV. In addition, their seroconversion even at the older age suggests that the more robust BALB/c and outbred strains likely will seroconvert effectively also.

Our data indicate that both C57BL/6 and BALB/c mice first exposed to MNV at 40 wk of age or older seroconvert equally as efficiently as young mice when inoculated directly and seroconvert equally well (BALB/c) or faster and more efficiently (C57BL/6) after indirect exposure to MNV-positive soiled bedding. Although these results do not guarantee that older sentinels will reliably seroconvert to other typical rodent

pathogens, they do support the possibility of reducing sentinel animal numbers by extending their residence from 3 to 12 mo. Additional studies addressing other common pathogens are needed to validate older mice as adequate sentinels. Other testing modalities (for example, RT-PCR analysis, exhaust duct sampling) can be combined with serologic methods to increase the sensitivity and specificity of rodent surveillance programs and minimize the use of live animals where possible.

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