ISOLATION, CHARACTERIZATION, AND DIAGNOSIS OF MURINE NOROVIRUSES, A NEWLY RECOGNIZED PATHOGEN OF MICE

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

ISOLATION, CHARACTERIZATION,
AND DIAGNOSIS OF MURINE NOROVIRUSES,
A NEWLY RECOGNIZED PATHOGEN OF MICE

presented by Charlie Chun Hsu,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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This work is dedicated to Dylan Yong Hsu, born November 27, 2006.

No matter where we move or where we live, through him,
we will always be reminded of the people we met and the friends we made
in Columbia, Missouri, his birthplace.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ISOLATION, CHARACTERIZATION, AND DIAGNOSIS OF MURINE NOROVIRUSES, A NEWLY RECOGNIZED PATHOGEN OF MICE

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ABSTRACT

Murine norovirus (MNV) is a newly recognized pathogen of mice that causes lethal infection in mice deficient in components of the innate immune response but no clinical disease in immunocompetent mice. To assess the extent of MNV infection in laboratory mice, serologic diagnostic assays including the high-throughput, multiplex fluorescent immunoassay (MFI), were developed and revealed that 22.1% of 12,639 serum samples from mice contained antibodies to MNV. These results indicate evidence of widespread MNV infection in research mice and make MNV the most prevalent viral pathogen in laboratory mice. In addition, a RT-PCR primer pair with a sensitivity of 25 virus copies was developed. These assays provide the necessary tools for diagnosis of MNV infection in laboratory mice to aid in the establishment of MNV-free mouse colonies.

Previously, murine norovirus 1 (MNV-1) was the only norovirus reported to infect research mice. We describe the isolation of 3 novel murine norovirus strains—MNV-2, MNV-3, and MNV-4—that were isolated from geographically separate mouse research colonies. All 4 murine norovirus strains used as individual antigens in the MFI displayed antigenic cross-reactivity to serum from mice inoculated with MNV-1, MNV-2, MNV-3, or MNV-4. These novel strains were also used to optimize the RT-PCR assay

and a primer pair was developed that detected all known MNV strains. Further studies indicated that these novel MNV strains caused persistent infections and prolonged fecal shedding in experimentally inoculated mice, which is markedly different in pathogenicity to MNV-1 which causes only transient infection lasting a few days. This is the first report of long-term infection and shedding of noroviruses in mice. Comparisons of the nucleotide and predicted amino acid sequences of the three novel MNV strains with each other, MNV-1, and other previously described human and animal noroviruses indicated that these three novel MNV strains were related to each other and MNV-1 even though they display markedly different biologic behavior from that of MNV-1.

In another study, we describe the use of microarrays, real-time RT-PCR and protein assays to document immunologic perturbations caused by MNV infection in BALB/c and C57BL/6 mice. No significant changes were identified in MNV infected mice of either mouse strain at the mRNA or protein level. The cytokines and chemokines evaluated encompassed over 100 genes involved in the inflammatory response. Further studies are warranted to determine the impact of MNV infection and their potential to act as a confounding factor in biomedical research when using MNV infected laboratory mice.

Finally, we show serologic evidence suggestive of a host-specific norovirus infecting rats. A Western blot assay using MNV proteins as the antigen revealed that a subset of rat serum samples highlighted the 59 kDa major antigenic capsid protein of noroviruses. These serum samples were also positive by the MNV MFI and indirect fluorescent antibody assay. However, no nucleotide sequence data or a cultivatable rat norovirus isolate could be obtained.

CHAPTER I

Introduction and Background

DISCOVERY AND HISTORY OF NOROVIRUSES

Epidemics of acute nonbacterial gastroenteritis in humans was first described in 1929 (237). Initially called "winter vomiting disease," this syndrome consists of a sudden onset of severe nausea and vomiting, although diarrhea may also be present or the predominant clinical sign. A viral etiology was suspected in these epidemics since none of the known bacterial causes of gastroenteritis could be isolated. However, even though a viral agent was suspected as the cause, standard viral culture methods to isolate and identify the virus were unsuccessful. During the 1940s and 1950s, numerous reports from the United States and Japan confirmed the experimental transmission of gastroenteritis to human volunteers by oral administration of bacteria-free fecal filtrates derived from patients with clinical symptoms in epidemic outbreaks (54, 103). In 1968, a notable outbreak of epidemic acute nonbacterial gastroenteritis occurred in an elementary school in Norwalk, Ohio, that caused illness in 50% of 232 students and teachers (3). Time of onset for the majority of cases occurred within a 24 hour period and clinical symptoms consisting of nausea, vomiting, abdominal cramps, lethargy, diarrhea, and fever typically lasted for 12 to 24 hours. As in previous cases, studies failed to reveal a bacterial or parasitic etiology in the Norwalk outbreak and a viral etiology was once

again suspected since bacteria-free filtrates from a rectal swab of an ill patient was able to induce gastroenteritis in human volunteers (35, 36). The causative agent could then be serially passaged in additional naïve human volunteers administered fecal filtrates derived from ill volunteers in previous rounds of inoculations (35, 36). It was not until 1972 when Kapikian et al. (102) used immune electron microscopy on a stool specimen from one of these ill volunteers to visualize a 27 to 32 nm particle associated with the disease. This particle was the first report of Norwalk virus, the prototype strain of a diverse group of viruses now called noroviruses. Although continued efforts to grow human noroviruses in cell culture failed, the Norwalk virus genome was successfully cloned and characterized in 1990 from human stool samples (236). This critical step allowed for the cloning of additional strains of noroviruses, the genetic classification of noroviruses, and the development of molecular diagnostic assays such as reverse transcriptase-polymerase chain reaction (RT-PCR). It would soon be revealed that noroviruses consist of a large group of genetically and antigenically diverse viruses infecting both humans and animals.

CLASSIFICATION AND MOLECULAR CHARACTERISTICS OF NOROVIRUSES

Previously referred to as "Norwalk-like viruses" or "small round structured viruses," noroviruses are nonenveloped, positive-sense, single-stranded RNA viruses with icosahedral symmetry (65, 143, 188, 218). The *Norovirus* genus is within the family *Caliciviridae*, which also includes the genera *Sapovirus*, *Lagovirus*, and *Vesivirus* (62). *Calici* is derived from the Latin word *calyx*, or cup, referring to the cuplike depressions

seen on the surface of the small 27 to 40 nm virions (63). A routine cell culture system to propagate human noroviruses has eluded researchers despite numerous efforts for many years (42). Only 3 recent reports have described the successful culture of human noroviruses using differentiated human embryonic intestinal epithelial cells grown on microcarrier beads or using T7 vaccinia virus expression systems (7, 109, 206). As a result, many of the characteristics of human norovirus infection and replication have been extrapolated from studies with related animal caliciviruses that are permissible in cell culture, such as the feline calicivirus, porcine enteric calicivirus, and the recently recognized murine norovirus (22, 24, 25, 41, 65, 153, 189, 203, 231), or from studies using human volunteers given norovirus infected fecal filtrates (4, 35-39, 57, 102, 127, 233).

Due to the lack of easily renewable virus by in vitro propagation, methods such as serotyping to characterize human noroviruses are difficult to perform. As a result, genetic analysis has been the predominant approach to classify norovirus strains. Although a uniform classification system for noroviruses has not been established (238), studies evaluating the phylogenetic relatedness of norovirus strains have divided the genus into 5 genetic groupings, or genogroups, based upon nucleotide or amino acid sequences of the entire, or portions of, the RNA dependent RNA polymerase or capsid regions of the genome (6, 59, 62, 74, 86, 106, 108, 128, 174, 187, 213, 219, 238). Human noroviruses are within genogroups I, II and IV (65, 238). Noroviruses infecting swine are within genogroup II, while noroviruses in cattle and mice are within genogroups III and V, respectively. A recently proposed classification scheme further divided noroviruses into 29 genetic clusters within the 5 genogroups (8 in genogroup I,

17 in genogroup II, 2 in genogroup III, 1 in genogroup IV, and 1 in genogroup V) (238), while another recent report suggested a 7 genogroup classification scheme with genogroups VI and VII containing human noroviruses (186). Strikingly, these studies revealed an enormous amount of genetic diversity between noroviruses in different genogroups, and even among norovirus strains within the same genetic cluster. For example, full-length nucleotide sequences comparing noroviruses in all 5 genogroups differ by as much as 47%, while within the same genogroup by as much as 26% (86). Predicted amino acid capsid sequences among noroviruses in all 5 genogroups vary by as much as 61%, while within the same genogroup by as much as 35% (86, 238). When comparing only norovirus strains that infect humans, they have differences as great as 44% and 58% in the full-length nucleotide and predicted amino acid capsid sequences, respectively (86, 238). The genetic diversity of norovirus strains may be due, in part, to the lack of proofreading activity of the viral RNA dependent RNA polymerase (43, 232). In addition, recombination has been reported to occur among noroviruses at the junction of open reading frames (ORF) 1 and 2 (5, 20, 73), providing another mechanism of generating genetically divergent viral strains.

The norovirus genome is approximately 7.5 kilobases, polyadenylated, and contains 3 ORFs (65). ORF1 encodes the nonstructural proteins that are formed through proteolytic cleavage of a large polyprotein by the virus-encoded 3C-like proteinase (65, 204). The nonstructural proteins of ORF1 include an N-terminal protein, NTPase, 3A-like protein, VPg, 3C-like proteinase, and RNA dependent RNA polymerase. Although the exact functions of the N-terminal protein and 3A-like protein are unknown, the VPg protein is covalently linked to the 5'-end of the viral RNA and may be important for

translation initiation (25, 33, 65, 204). ORF2 encodes the major capsid protein, also call viral protein 1 (VP1), which assembles to form the capsid (188). The capsid protein can be divided into 2 domains, the S domain which forms the shell of the capsid, and the P domain forming protrusions from the shell (188). The P domain is further subdivided into the P1 and P2 subdomains. The amino acid sequences of the S domain are the most conserved within VP1 while the P2 subdomain is highly variable since it is the most exposed portion of the protein involved with cellular binding and immunologic reactivity (130, 188, 210, 212). ORF3 encodes a small basic protein, or VP2, which is important for the stability and increased expression of VP1 (12). A VPg-linked 2.3 kilobase subgenomic RNA containing ORF2, ORF3, and the 3' untranslated region is also produced in abundance during norovirus replication (7, 12, 109, 230, 231). The subgenomic RNA, rather than the genomic RNA, is used as the template for the production of the capsid proteins (7, 12, 65). Additionally, the 5' end of the subgenomic and genomic RNAs contain a highly conserved nucleotide motif upstream from the first AUG start codons and is therefore thought to be a site for the initiation of transcription (86, 153).

NOROVIRUS INFECTIONS IN HUMANS

Noroviruses are the major cause of human epidemic nonbacterial gastroenteritis worldwide (52, 65). In the United States, it is estimated that noroviruses cause 23 million illnesses, 50,000 hospitalizations, and approximately 67% of all food-related illnesses due to known pathogens each year (146, 229). Noroviruses account for greater than 95% of outbreaks of nonbacterial gastroenteritis (45, 46) and are classified as a category B

pathogen with bioterrorism potential by the National Institute of Allergy and Infectious Disease.

Disease due to norovirus infection can occur in both adults and in children, although rotaviruses must also be considered in cases of acute gastroenteritis in pediatric patients (16, 29, 30, 92, 147, 163, 177). Clinical symptoms include vomiting, non-bloody diarrhea, fever, abdominal cramps, nausea, chills, myalgia, and headaches (35, 65, 105); however, asymptomatic infections after experimental inoculations have been described (57). In children, vomiting has been reported to occurred more frequently than diarrhea (3, 56, 105) although in some outbreaks others have reported a higher prevalence of diarrhea in children (195). Symptoms of norovirus infection are typically considered mild and self-limiting, lasting for 24 to 48 hours, with an incubation period of 24 to 48 hours after exposure and prior to clinical disease (35, 36, 54, 57, 65, 104, 105). Medical intervention is generally not required unless severe illness is induced, such as in infants, the elderly or immunosuppressed patients, in which case fluid therapy or additional supportive care may be needed (65, 178, 229). A malabsorptive diarrhea has been suggested since adenylate cyclase activity is not increased during infection and noroviruses cause a malabsorption of fat, D-xylose, and lactose in the small intestines of experimentally inoculated human volunteers (14, 125, 199). At the microscopic level, small intestinal biopsies collected during experimental infections in human volunteers with the prototypic Norwalk virus, a genogroup I norovirus, or with Hawaii virus, a genogroup II norovirus, displayed an intact mucosa with blunting and broadening of villi, shortening of the microvilli, mononuclear and polymorphonuclear leukocyte inflammation in the lamina propria and between epithelial cells, crypt hypertrophy with

increased epithelial cell mitoses, and vacuolated epithelial cells (4, 38, 199, 200). Similar to the short duration of clinical symptoms, these microscopic lesions resolved rapidly, within 2 weeks after clinical illness (4).

Outbreaks of norovirus infection commonly occur in areas of close contact which aids in the spread of the virus, such as in restaurants, hotels, hospitals, cruise ships, schools, recreational camps, day cares, nursing homes, and military vessels (1, 46, 47, 64, 93, 104, 105, 135). A predominant seasonal occurrence of outbreaks during the winter months has been reported and thus the initial description of "winter vomiting disease," but cases of norovirus infections peaking in the spring have also been described (64, 79, 155). Transmission of noroviruses can occur from person to person, by the fecal-oral route, through contaminated foods, water or surfaces, or as suggested by epidemiological studies, through aerosolized vomitus (54, 135, 136, 142, 229). Norovirus outbreaks have been associated with a variety of contaminated foods including fruits, salads, cold meats, bakery products, cake frosting, and shellfish (50, 51, 65, 229). Most notably, shellfish such as clams and oysters may accumulate noroviruses when grown in fecal waste-contaminated waters and it has been suggested based on epidemiologic studies that cooking or steaming contaminated shellfish prior to consumption does not prevent contracting norovirus induced illness (118, 144, 201).

It was originally thought that infected individuals only shed virus for a few days after the onset of clinical illness as detected by immune electron microscopy (214). However, as more sensitive diagnostic assays were developed, it was revealed that while the amount of virus excreted peaks a few days after infection, shedding may last for up to 3 weeks in some patients to greater than 6 weeks in infants (57, 135, 158, 173, 195, 214).

Immunosuppressed individuals, such as transplant recipients on immunosuppressive therapy, may have persistent diarrhea and shed infectious virus for years (110, 168).

In the last 10 years, there has be the emergence and predominance of genogroup II, genetic cluster 4 noroviruses causing outbreaks of acute nonbacterial gastroenteritis around the world (17, 21, 46, 65, 119, 170, 172, 228). Another recent report describes the predominance of genogroup II, genetic cluster 3 noroviruses in pediatric patients in Japan from 2004 to 2005 (186). To date, the mechanisms which allow specific norovirus groups to predominate in outbreaks are not fully understood.

STABILITY AND DISINFECTION OF NOROVIRUSES

The implication that elevated temperatures, such as those reached during the cooking and steaming process, are not effective to inactivate noroviruses is exemplary of the stability of these hardy viruses. Continued infection and illness, even after aggressive cleaning and control efforts were employed, in newly arriving hotel guests over a 6 month period (27) and on a single cruise ship for 6 consecutive cruises (71, 93, 228) further exemplifies the durability of these viruses. Dolin et al. reported that exposing noroviruses in fecal filtrates to 20% ether for 24 hours at 4°C, a pH of 2.7 for 3 hours at room temperature, or 60°C for 30 minutes, did not inactivate Norwalk virus or prevent illness in challenged volunteers (36). Keswick et al. showed that standard chlorination levels in drinking water (0.5 to 2 ppm) does not prevent norovirus infection in human volunteers and that at least 10 mg/L (10 ppm) of chlorine is required to prevent symptomatic infections (114). Other studies on norovirus stability and disinfection have used in vitro assays with surrogate viruses, such as the feline calicivirus (40, 41) or the

recently discovered murine norovirus (22, 117, 179), since these viruses grow in standard cell culture. In these studies, sodium hypochlorite concentrations of at least 5000 ppm for 1 minute (40) or 3000 ppm for 10 minutes (41) were considered effective to inactivate feline calicivirus. As a frame of reference, ready to use commercial bleach solutions typically contain 5% sodium hypochlorite, equivalent to 50,000 mg/L (or ppm); a 1:10 dilution would contain 5000 ppm of sodium hypochlorite. Glutaraldehyde and iodine based products were also found to be effective (40). When viruses were exposed to temperatures of 63°C or greater, rapid reductions of infectious virus were seen within the first few minutes (22, 40, 41). However, these surrogate viruses were resistant to disinfection by quaternary ammonium products, organic solvents, 75% ethanol, and sodium hypochlorite at 300 ppm or less (22, 40, 41). Additionally, caliciviruses were minimally affected when stored at 4°C, although at room temperature the amount of infectious virus gradually decreased in a time dependent manner over the span of 1 to 2 weeks (22, 40, 41). Collectively, the combination of the stability of noroviruses, the low infective dose of less than 100 viral particles to induce infection (52, 91, 126, 150, 178), and the large amount of virus excreted from infected individuals (10^4 to 10^9 viral copies per gram of feces) (52, 176) may explain, in part, the epidemic outbreaks of norovirus gastroenteritis that occur in humans.

DIAGNOSIS OF NOROVIRUS INFECTIONS

Development of diagnostic assays to detect norovirus infections in humans has been hampered by the lack of a simple cell culture system to propagate virus (42). As a result, early diagnostic assays for norovirus infection relied upon reagents collected from

human volunteers who developed illness after being given fecal filtrates containing infectious virus (102). Direct electron microscopy can be used to detect enteric viruses, however this method is rather insensitive since a concentration of at least 10^6 virus particles per ml of stool is required and noroviruses are usually present in low concentrations (8, 65). An improvement over direct electron microscopy is immune electron microscopy, which was the technique used to first detect Norwalk virus in a fecal sample from an infected human volunteer and implicate its role as the causative agent of acute nonbacterial gastroenteritis (102). However, while an improvement over direct electron microscopy, immune electron microscopy was still rather insensitive and unable to detect virus in a significant number of experimentally infected volunteers (214). Over time, other diagnostic tests were developed such as radioimmunoassays, enzyme immunoassays or Western blot assays to detect either norovirus antigen or antibodies (8, 47, 66, 78, 81), but some of these assays still relied upon reagents purified from a limited amount of sera and infected stool samples from human volunteers.

The successful cloning of the Norwalk virus genome (140, 236) marked the next generation of diagnostic assays. Molecular assays such as reverse transcriptase-polymerase chain reaction (RT-PCR) could now be developed to detect Norwalk virus in fecal samples from outbreaks of nonbacterial gastroenteritis (95, 151). However, as new strains of noroviruses were discovered, sequenced, and characterized, it was soon revealed that these viruses exhibit a great amount of genetic diversity (108). Luckily, conserved regions within multiple areas of norovirus genomes have been found such as in the polymerase gene and the ORF1/ORF2 junction (6, 8, 101, 123, 213). Therefore, broadly reactive PCR primer pairs capable of detecting multiple strains of human

noroviruses have been developed to diagnosis infection (98, 123, 220). To quantify the number of virus copies in a given sample, real-time RT-PCR assays have also been described (101, 176, 194).

An additional advance in the diagnosis of norovirus infections stemming from the cloning of the Norwalk virus genome is the capability to produce virus-like particles (VLPs) (60, 96). The structural protein encoded by ORF2, expressed in the baculovirus system, self-assembles to form empty VLPs similar to native viral capsids. Expression of ORF2 in conjunction with ORF3 and the 3' untranslated region has been described to increase the yield and stability of the recombinant VLP (12). These VLPs provide a renewable reagent that can be used for the production of hyperimmune sera in laboratory animals or for the development of either antigen or antibody detection assays for noroviruses (58, 61, 96, 97, 99). However, as was seen with the great genetic diversity of noroviruses, studies using VLPs have revealed a great antigenic diversity among noroviruses as well (11, 61, 97). Therefore, the diagnosis of norovirus infection in humans still remains a challenging task since no single primer pair or virus-like particle can be use to detect all strains of noroviruses circulating in humans.

IMMUNITY AND SUSCEPTIBILITY TO NOROVIRUSES

Studies of the immune response to norovirus infection have been challenging since human noroviruses cannot be grown in standard cell culture and they do not infect a multitude of experimentally inoculated animal species (36, 42). Additionally, only pigs and cattle have been previously reported to harbor their own host-species specific norovirus (73, 221) making these large animals the only available animal models to study

the disease. Recently, a human norovirus has been propagated in a 3-dimensional organoid model of human small intestinal epithelium (206) and a mouse model of norovirus infection have been reported (106). Even though the host-specific norovirus infection in mice does not cause gastroenteritis (86, 231) and it is not know if the in vitro organoid model will support the growth of all strains of human noroviruses, these advances may nonetheless prove useful in future studies on the immunologic response to norovirus infection. As a result, most of the current knowledge on the immune response to norovirus infection has been derived from natural outbreaks or experimental studies using human volunteers challenged with infected fecal filtrates or with empty capsid VLPs (57, 127, 209).

Experimental studies in humans with symptoms of acute viral gastroenteritis have shown that a transient lymphopenia occurs during illness associated with infiltration of the small intestines with lymphocytes and polymorphonuclear cells (4, 38, 39). Although an early report in the literature failed to identify any changes in interferon expression in jejunal biopsies, jejunal aspirates, or the sera of human volunteers experimentally inoculated with either Norwalk or Hawaii virus (37), other studies from natural outbreaks or experimental norovirus infections have provided some insight on the cytokine response in humans. In one study of a natural outbreak of diarrhea in U.S. students travelling to Mexico, increases in fecal interleukin (IL)-2 and gamma interferon (IFN-γ) were found in the absence of fecal IL-4, IL-5, and IL-10 suggesting a predominantly T helper (Th) type 1 immune response to norovirus infection (120). Likewise, after experimental inoculation in human volunteers with Snow Mountain virus, a genogroup II norovirus, increases of IFN-γ and IL-2, but not IL-4, IL-6 or IL-10, were found in the

serum or in supernatants of peripheral blood mononuclear cells stimulated with recombinant VLPs (127). In volunteers immunized with Norwalk VLPs, increases of IFN-γ in the absence of IL-4 production was seen in peripheral blood mononuclear cells stimulated with Norwalk VLPs, again supporting a Th1 response (209). Gnotobiotic pigs that develop mild diarrhea after inoculation with human noroviruses exhibit increases of IFN-α, IL-12 and IFN-γ, however they also express low levels of IL-4, IL-6 and IL-10 suggesting that a Th2 response may also be present (205).

Seroprevalence studies in humans have identified antibodies to noroviruses in greater than 90% of people tested in some areas, indicating that norovirus infections are quite common worldwide (131, 165, 183). Increases in serum IgG, serum IgA and fecal IgA antibodies have been described in ill individuals from both natural outbreaks and human volunteer challenge studies, but seroconversion has also been reported in asymptomatically infected humans (15, 38, 57, 100, 102, 169, 173, 196). Studies with human volunteers given bacteria-free fecal filtrates containing Norwalk virus have shown that short-term immunity develops in those who become ill following challenge since rechallenge with the homologous virus 6 to 14 weeks later (or as long as 6 months later) failed to induce illness (36, 100, 141, 182). Likewise, cross-challenge studies have shown that those given a different but antigenically related norovirus strain 6 to 14 weeks after the initial Norwalk challenge are protected from norovirus induced disease (233). However, long term immunity is not induced since some volunteers became ill when rechallenged with Norwalk virus 27 to 42 months after the initial challenge (141, 182). It is unclear at this point what role antibodies play in regard to the prevention of and the resistance to norovirus infection since paradoxically, those with preexisting antibody

titers were more likely to develop clinical illness than those with low antibody titers who were resistant to infection (15, 57, 100, 141, 173, 182). These resistant individuals failed to develop a serologic response even after multiple challenges (100, 182). However, this finding is not universal since some volunteers that lack preexisting antibody still develop symptomatic infections (57). Nonetheless, since there is evidence that short-term immunity develops after infection and it has been suggested that a rapid mucosal IgA response, such as from a memory response, may be associated with preventing infection (126), vaccination strategies using recombinant VLPs in attempts to prevent norovirus induced illness are currently under investigation (10, 44, 70, 76, 166, 185).

Recent evidence indicates that susceptibility and resistance to norovirus infection is determined by the carbohydrate binding properties of the virus to human histo-blood group antigens (HBGAs) and that these binding properties may help explain the uncertainties about protective immunity. Human HBGAs are complex carbohydrates linked to glycoproteins or glycolipids in the cellular membranes of red blood cells and epithelial cells of tissues exposed to the external environment (e.g. gastrointestinal, upper respiratory, and lower genitourinary tract) (91, 124, 133). Also, HBGAs can be expressed in a secreted form in saliva, milk and intestinal contents. The most familiar of these antigens are the human ABO blood groups; however the HBGAs include other components called H and Lewis antigens. Many pathogens including viruses, bacteria, and protozoa use these or other carbohydrates (e.g. heparan sulfate or sialic acid) expressed on host cells and tissues as receptors for binding and attachment during infections (91, 124). While noroviruses do not utilize heparan sulfate or sialic acid as receptors like many other viruses do, studies with norovirus VLPs have shown the

importance of the HBGAs in determining the host susceptibility or resistance to norovirus infection (90, 126).

The human HBGAs are produced by a number of enzymatic reactions that occur during their biosynthesis. These enzymes, a group of glycosyltransferases, add monosaccharide units to disaccharide precursors to produce a number of different but related tri- and tetra-saccharide units. Two of these glycosyltransferases encoded by the genes *FUT1* and *FUT2*, add a fucose onto precursors to produce the H type 2 and H type 1 antigen, respectively. Next, the H antigens can be further modified by the A and/or B enzymes which add another monosaccharide to produce the A, B or AB antigens of the familiar red blood cell groups. Those without functioning A and B enzymes only express the H antigen and correspond to an O blood type. Finally, the *FUT3* gene is responsible for the addition of another fucose, distinct in position from the fucose added by the FUT1 or FUT2 enzymes, to produce the Lewis antigens. Therefore, the variety of which of these tri- and tetra-saccharide moieties get produced and expressed on the distal ends of the carbohydrate chains linked to glycoproteins and glycolipids is dependent upon a person's genetics and expression of these glycosyltransferases. For example, those lacking expression of the *FUT2* gene are considered "nonsecretors" since this enzyme is responsible for expression of the ABH antigens in epithelial cells of the gut and secreted bodily fluids such as saliva. The presence or absence of the *FUT3* gene determines whether an individual is "Lewis positive" (Le^+) or "Lewis negative" (Le^-), respectively. Additionally, as cells differentiate and mature from the crypts to the villus tips of the small intestinal epithelium, they can express a greater variety and higher amounts of carbohydrates including the ABH and Lewis antigens.

Initially, studies with rabbit hemorrhagic disease virus (RHDV), a calicivirus in the genus *Lagovirus*, showed the importance of the H antigen in viral attachment and binding to rabbit epithelial cells (198). Therefore, this observation prompted studies to evaluate if HBGAs were important for Norwalk virus binding and attachment. Using red blood cells, gut epithelial cells, saliva or Caco-2 cells incubated with recombinant Norwalk VLPs, it was revealed that Norwalk VLPs bound efficiently to carbohydrates of secretor positive individuals (expressing the *FUT2* gene) with blood types A, AB and O, but less efficiently to B blood type individuals (75, 90, 134). These results suggested that type B individuals might be resistant to infection with Norwalk virus. Additionally, no binding was observed in samples from secretor negative individuals (those lacking *FUT2* gene expression) (75, 90, 134). To support these findings in vivo, human volunteers with type B histo-blood antigens experimentally challenged with Norwalk virus were more resistant to infection than those of the other blood types since only a small percentage of type B individuals were infected and became symptomatic; nonsecretor individuals did not become infected at all (89, 126). The importance of the FUT2 enzyme in norovirus infection was again shown, this time in a natural outbreak setting with a genogroup II norovirus, when only secretor individuals with a functional FUT2 enzyme, and none of the nonsecretor individuals, had symptomatic infections (215). However, as more norovirus strains were identified and evaluated, it became evident that a wide variety of binding patterns existed that were dependent on the hosts ABH, Lewis, and secretor status (77, 88, 124, 211). For example, while Norwalk VLPs bind efficiently to A, AB and O secretors, VLPs from norovirus strains Grimsby, VA387 and Dijon95 are able to bind to secretor antigens of all blood types, while VLPs of strains VA207, Boxer and OIF

bound only to nonsecretor samples (87, 88, 124). In a study of 14 noroviruses, Huang et al. described 7 different binding patterns that could be classified into two groups: the A/B binding group which bound to type A and/or B and O antigens of secretors but not to nonsecretors, and the Lewis binding group which bound to nonsecretors and type O secretors with weak binding or no binding to type A and B secretors (88). Interestingly, the carbohydrate binding properties of different noroviruses do not segregate with the genogroup classifications of noroviruses (88, 211). Thus, it would be difficult to predict which groups of people will be susceptible to a specific norovirus strain during an outbreak based solely on the genetics and classification of the virus.

Although the discovery of the HBGAs and their role in norovirus binding, attachment and symptomatic infections was a significant breakthrough, additional components of the infectious process beyond carbohydrate binding need to be elucidated since Caco-2 cells, which express the proper HBGAs and bind recombinant Norwalk VLPs (227), are not permissive to Norwalk virus infection (42). Also, there are some strains of noroviruses whose corresponding VLPs do not bind any HBGAs tested (88) suggesting that other mechanisms of attachment may exist. However, as discussed in a review by Hutson et al. (91), the carbohydrate binding properties of noroviruses may help explain why individuals without pre-existing antibodies to noroviruses doesn't necessarily predict susceptibility to infection. These individuals may lack the necessary carbohydrates required for norovirus attachment and entry and consequently they do not become infected with and produce antibodies to noroviruses. In contrast, having pre-existing antibodies may indicate previous infection and thus susceptibility, in which case

protective immunity will be dependent on the time after infection, number of previous exposures to induce immunity, or exposure to antigenically related strains.

NOROVIRUSES IN ANIMALS

In order to try and develop an animal model to study norovirus pathogenesis, attempts to inoculate multiple animal species with human strains of noroviruses have been ultimately unsuccessful. Only a few reports have described infection, seroconversion, and the development of diarrhea in a subset of animals inoculated with human noroviruses, but these required the use of immunocompetent gnotobiotic pigs or immunoincompetent newborn pigtail macaques (28, 205, 207). Other species that have been inoculated with human noroviruses but do not develop any clinical symptoms of disease include mice, rabbits, guinea pigs, kittens, calves, baboons, common marmosets, cotton top tamarins, owl monkeys, patas monkeys, cebus monkeys, cynomolgous monkeys, rhesus monkeys, and chimpanzees; although seroconversion was reported in chimpanzees and a rhesus monkey suggesting asymptomatic infection occurred in these animals (36, 65, 197, 234, 235). A seroprevalence study conducted at a U.S. primate research center indicated that sera from a large proportion of mangabeys, pigtail macaques, rhesus macaques and chimpanzees contained cross-reactive IgG antibodies to human noroviruses suggesting that these non-human primates may harbor their own host-specific norovirus (94). However, correlation with diarrhea was not evaluated and isolation of a norovirus from a non-human primate has not been reported to date.

While the family *Caliciviridae* includes viruses that infect a wide range of animal species such as cats, dogs, rabbits, sea lions, pigs, cattle, reptiles, amphibians, fish and

birds (40, 49, 63, 128, 138, 139, 149, 181), noroviruses have only been found in cattle, pigs and mice (106, 174, 193, 221). Recently, a norovirus in a lion cub related to human genogroup IV noroviruses was detected by molecular assays, but it is unknown whether this is a novel norovirus in a newly reported host species or an incidental finding of a human norovirus in the captive lion cub (137). Diarrhea due to norovirus infection is not typically observed in pigs and mice (86, 208, 222, 223), however diarrhea in calves has been reported in both natural and experimental norovirus infections (32, 128). Although no evidence of zoonotic transmission has been reported and noroviruses are considered to be host-species specific (63, 174, 217), it has been hypothesized that norovirus infections in animals could potentially serve as a reservoir for zoonotic transmission or viral recombination events leading to new emerging strains (73, 186, 217, 221, 222).

MURINE NOROVIRUS

The discovery of a norovirus infecting mice was described for the first time in 2003 from laboratory mice deficient in both the recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT1) (RAG2/STAT1$^{-/-}$ mice) (106). These severely immunocompromised mice sporadically succumbed to a multi-systemic disease from an unknown pathogen since testing of these mice for many of the known human or mouse pathogens were negative (231). A virus was suspected as the etiologic agent since disease could be serially passaged by intracerebral inoculation with diseased brain homogenates passed through a 0.2 μm filter. Both RAG2/STAT1$^{-/-}$ mice and mice lacking the alpha/beta and gamma interferon receptors (IFNαβγR$^{-/-}$ mice) intracerebrally inoculated with the unknown pathogen succumbed to infection within 30

days. Encephalitis, cerebral vasculitis, meningitis, hepatitis and pneumonia were reported in these mice. To identify the pathogen, representational difference analysis was performed and a nucleotide sequence related to known noroviruses was obtained. A combination of PCR and rapid amplification of cDNA ends was used to uncover the entire nucleotide sequence which was found to contain many characteristics similar to other noroviruses. Phylogenetic analysis further confirmed its identity as a novel norovirus and this initial strain of a norovirus infecting mice was designated murine norovirus 1 (MNV-1).

To deduce the immunologic factors involved during norovirus infection, mice lacking a variety of components of the immune system were inoculated with MNV-1 and evaluated (106). Unexpectedly, severely immunocompromised mice lacking both T and B cells of the adaptive immune response ($RAG1^{-/-}$ and $RAG2^{-/-}$ mice) survived after MNV-1 inoculation. These results suggested that an intact adaptive immune system was not necessary to prevent lethality. However, since immunocompetent strain 129 and C57BL/6 mice cleared MNV-1 infection after inoculation but $RAG^{-/-}$ mice remained persistently infected, it was proposed that T and B cells are required to clear the viral infection. To evaluate the innate response to MNV infection, mice lacking components of the innate immune system were infected with MNV-1. Mice lacking IFNαβ receptors, IFNγ receptors, inducible nitric oxide synthase (iNOS), or protein kinase RNA-activated (PKR), were all resistant to MNV-1 induced lethal infection. Alternatively, mice without a functional STAT1 ($STAT1^{-/-}$) alone, or in combination with a nonfunctional PKR ($STAT1/PKR^{-/-}$), succumbed to lethal MNV-1 infection similar to $RAG2/STAT1^{-/-}$ mice and $IFN\alpha\beta\gamma R^{-/-}$ mice described previously. Collectively, these data indicate that the

STAT1-dependent innate immune response, which is involved with signaling through the IFNαβ and IFNγ receptors, is required for resistance to lethal MNV-1 infection. Also, these data indicate that the IFNαβ and IFNγ receptors can compensate for one another since mice lacking either receptor alone were resistant to lethal infection but mice lacking both receptors were susceptible.

As new strains of MNV in laboratory mice were isolated and sequenced, it was soon revealed that like human noroviruses, noroviruses in laboratory mice contain a large number of genetically diverse strains (156, 213). The murine norovirus strains that have been isolated and sequenced to date are related to each other with full-length nucleotide identities ranging from 87.0 to 94.1%. In contrast, human norovirus strains may have nucleotide identities as low as 51% (108). In a study evaluating 26 full-length MNV genomes, the first 32 nucleotides at the 5' end, the 64 nucleotides at the ORF1/ORF2 junction, and the 47 nucleotides in ORF 2 were completely conserved among all strains (213). These 26 MNV genomes have been categorized into 15 distinct strains within a single genogroup, genotype and serotype (213). As additional strains of MNV are isolated and sequenced, it will be interesting to see if new serotypes, genogroups, or genotypes are identified. Evidence of recombination has been described among MNV genomes (156, 213) suggesting that like human noroviruses, noroviruses infecting mice are continually evolving and therefore additional genetically divergent strains of MNV likely exist.

Although the first norovirus strain, MNV-1, was rapidly cleared from infected immunocompetent mice (106, 213), subsequent studies using newly isolated field strains of MNV have shown that in great contrast to infection with MNV-1, all field strains with

one exception (WU11), cause persistent infections in immunocompetent mice (213). Also, mice infected with field strains of MNV shed infectious virus in the feces for prolonged periods and infection can be transmitted by soiled bedding to naïve sentinel mice (184, 213). Clinical disease such as diarrhea attributable to MNV infection has not been observed in immunocompetent mice, many immunodeficient strains, or in neonatal mice (106, 156, 157, 184, 213). Likewise, histopathologic lesions have not been typically seen in immunocompetent mice infected with MNV, except in two reports describing red pulp hypertrophy and white pulp activation in the spleen, mild granulocytic infiltrates in the intestines, and few hepatic inflammatory foci (157, 184). The splenic changes were attributed to increases in macrophages and B-cells since increased expression of F4/80 and B220 with only a modest increase in CD11c (the pan-murine dendritic cell marker) were found (157). However, the intestinal inflammation and splenic changes seen by histopathology were only observed at 24 hours and 72 hours, respectively, and not at any other time point evaluated, making interpretation of the significance of these findings difficult. Furthermore, the few hepatic inflammatory foci reported during natural MNV infection were not always seen in association with MNV antigen and these lesions are a common finding in the livers of mice (184). Also, no hepatic lesions were seen by histopathology in another report describing strain 129 mice experimentally infected with MNV-1 (157). Collectively, these data suggest that these previously reported lesions attributed to MNV may not be characteristic of MNV infection in immunocompetent mice. In immunodeficient mice, histopathologic lesions have been seen in some strains of both experimentally and naturally infected mice and consisted of hepatitis, focal interstitial pneumonia, pleuritis and peritonitis (106, 157,

225). However, while experimental MNV infection in $STAT^{-/-}$ and $IFN\alpha\beta\gamma R^{-/-}$ have demonstrated multisystemic histopathologic lesions (106, 157), it is unclear if the lesions observed in naturally infected immunodeficient mice in one report are indeed caused by MNV or rather just associated with MNV infection since other pathogens (e.g. *Helicobacter* sp.) were also present in these colonies of mice (225).

Immunohistochemical studies of the liver, spleen, mesenteric lymph node, and intestines have revealed that MNV has a tropism for macrophages and dendritic cells (157, 184, 225, 230). Detection of the nonstructural proteins, indicative of viral replication, in wild-type mice was found in the lamina propria (which contain macrophages and dendritic cells) of the intestines but not in epithelial cell (157). MNV nonstructural proteins have been observed in the epithelial cells of $STAT1^{-/-}$ and $RAG1/STAT1^{-/-}$ mice early in infection (12 hours PI), but by 24 to 72 hours PI, the nonstructural proteins localized to the lamina propria and Peyer's patches, and were seen only infrequently in epithelial cells (157). Therefore, although noroviruses are enteric pathogens, it is unclear at this point if epithelial cells are the primary routes of entry for MNV in immunocompetent mice. Likewise, the primary site of replication of human noroviruses has not been definitively determined but is thought to be in the upper intestinal tract based on lesions seen in biopsies from norovirus challenged human volunteers (4, 65, 199, 200). Whether noroviruses use small intestinal epithelial cells or dendritic cells capable of sampling the intestinal lumen by forming transepithelial dendrites (167, 231) as the primary route of infection has yet to be determined.

Based on the cellular tropisms identified by the immunohistochemical studies, MNV-1 was found to propagate and form plaques in RAW 264.7 cells, an immortalized

mouse macrophage cell line, providing the first report of in vitro propagation of a norovirus (230). However, variability in plaque formation or the capacity to produce a cytopathic effect in RAW 264.7 cells has been described in some MNV strains, even though all MNV strains isolated thus far are within the same genotype and serotype (213). Nonetheless, the discovery of a permissive cell line to propagate MNV in vitro has provided an important tool for the study of norovirus biology and pathogenesis. Since this was the first report of a norovirus to replicate in cell culture, previous studies were required to use other caliciviruses that could be propagated in cell culture, such as the respiratory feline calicivirus, and thus may not as accurately represent the biology of enteric noroviruses. To date, MNV has been used to study the translation initiation factors, proteolytic processing, and disinfection of noroviruses (22, 33, 117, 179, 204), and a reverse genetics system for MNV has been recently described (26, 226). Future studies using MNV will likely prove useful in analyzing norovirus pathogenesis and biology.

The remainder of this dissertation describes the additional advances made by our laboratory to understand the characteristics of MNV infection and biology in laboratory mice. In Chapter II of this dissertation, we take advantage of the in vitro permissibility of MNV-1 in RAW 264.7 cells to describe the development of both serologic and molecular diagnostic assays to detect MNV infection in laboratory mice. Notably, we describe the development of a novel, high-throughput, serologic assay termed the multiplex fluorescent immunoassay which uses small 5.6 μm microspheres coated with MNV-1 antigen. We used this assay to screen 12,639 serum samples from laboratory mice in the United States and Canada for antibodies to MNV. We were the

first to report widespread serologic evidence of MNV infection in laboratory mice, documenting that 22.1% of these sera were positive for MNV antibody (84). In Chapter III, we describe the isolation of 3 novel field strains of MNV—MNV-2, MNV-3, and MNV-4—and the refinement of our serologic and molecular diagnostic assays to include detection of all known strains of MNV. Notably, we were the first to report that all of these novel field strains of MNV, in great contrast to the previously reported MNV-1, caused persistent infections and prolonged fecal shedding in laboratory mice (85). In Chapter IV of this dissertation, we describe the molecular characteristics and phylogenetic analysis of these novel MNV strains with MNV-1 and other human and animal noroviruses. We confirm through molecular and phylogenetic analysis that these novel MNV strains are indeed noroviruses related to MNV-1 even though their biological behavior was markedly different from that of MNV-1 (86). In Chapter V, we attempt to identify the immunologic response that occurs in MNV infected immunocompetent BALB/c and C57BL/6 mice using microarrays, quantitative real-time RT-PCR, and protein detection assays. These studies were performed to determine the impact of MNV infection as a potential confounding factor in biomedical research studies using infected mice, as well as to elucidate the components of the immune response to MNV infection. We observed minimal alterations in inflammatory gene expression caused by MNV infection in mice after both acute and chronic infections. However, the significance and reliability of these findings are also discussed. Finally, in Chapter VI of this dissertation, we describe the search for a novel norovirus infecting laboratory rats and show serologic evidence that rats may harbor their own host-adapted norovirus.

CHAPTER II

Development of a Microsphere-Based Serologic Multiplexed Fluorescent Immunoassay and Reverse Transcriptase PCR Assay to Detect Murine Norovirus 1 (MNV-1) Infection in Mice

INTRODUCTION

Members of the genus *Norovirus* are nonenveloped viruses with a linear, positive-sense, single-stranded RNA genome (63). Noroviruses are in the family *Caliciviridae* which also include the genera *Sapovirus*, *Lagovirus* and *Vesivirus* (63, 143). Formerly known as "Norwalk-like viruses" or "small round structured viruses," noroviruses cause acute gastroenteritis in humans typically lasting 24 to 48 hours and infect people of all ages (63). Outbreaks occur all over the world and have been reported to occur in schools, nursing homes, hospitals, restaurants and cruise ships (34, 45, 79, 92, 121). The virus is shed in the feces and vomit, and is transmitted by exposure to infected individuals or contact with contaminated foods, water or surfaces. In addition, noroviruses have a very

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low infective dose of less than 100 viral particles, are persistent in the environment, and require at least 10 mg/L of chlorine to be inactivated (52, 114, 150, 178). Noroviruses are considered to be one of the most important causes of acute nonbacterial gastroenteritis in the world and have been detected in as high as 94% of outbreaks (45, 46). In addition, noroviruses account for an estimated 33 to 67% of all foodborne related cases of gastroenteritis (34, 72, 146, 229).

Recently, the first murine norovirus, murine norovirus 1 (MNV-1), was isolated from mice lacking recombination-activating gene 2 and signal transducer and activator of transcription 1 (RAG2/STAT1$^{-/-}$ mice) (106). Mice lacking the alpha/beta and gamma interferon receptors or STAT1 succumbed to MNV-1 infection after oral inoculation while wild-type 129 mice were asymptomatic. STAT1$^{-/-}$ mice showed histopathologic signs of pneumonia, liver fibrosis and loss of splenic architecture after 3 or 7 days post-infection. In addition, virus was detected in multiple organs, including the intestines, by quantitative real time reverse transcriptase PCR (RT-PCR) and was shed in feces. Subsequent studies identified a tropism of MNV-1 for murine macrophages and dendritic cells, including the murine macrophage cell line RAW 264.7 which is used for propagation of MNV-1, providing the first report of *in vitro* cultivation of a norovirus (230).

In this report, we describe the development of a high-throughput microsphere-based, flow cytometric serologic assay to screen mouse sera for anti-MNV-1 antibodies. Use of this assay to screen sera from mice in research colonies in North America demonstrated that infection with MNV-1 is widespread. In addition, a RT-PCR assay

specific for the detection of MNV-1 in fecal and tissue samples was developed as a method for identifying MNV-1 infection in mice.

MATERIALS AND METHODS

Propagation of MNV-1. Murine norovirus 1 (MNV-1.CW1, passage 3) was a kind gift from Herbert W. Virgin, Washington University School of Medicine (St. Louis, Mo). MNV-1 was grown as previously described with some modifications (106). RAW 264.7 cells (TIB-71; American Type Culture Collection, Manassas, Virginia) were maintained in Dulbecco's modified Eagle's medium (DMEM) (SH30243.02; HyClone, Logan, Utah) supplemented with 10% low-endotoxin fetal bovine serum (14-501F; Cambrex, East Rutherford, N.J.), 10 mM HEPES, and 10 µg/ml of ciprofloxacin. For large virus preparations, RAW cells were cultivated in 1-L MagnaFlex suspension spinner flasks (Wheaton Science Products, Millville, N.J.) and infected with MNV-1 when the cell count was approximately 1×10^6 cells/ml at a multiplicity of infection of 0.1. Cell viability was monitored by trypan blue exclusion assay and after 36 to 48 hours, when cells displayed 90 to 100% cytopathic effect (CPE), cellular material was pelleted at 2000 × g for 10 min at 4ºC and the clarified supernatants containing virus were frozen at -80ºC or immediately concentrated and purified.

MNV-1 concentration and purification. Viral particles were precipitated and concentrated by adding 1 M NaCl and 8% wt/vol of polyethylene glycol 8000 (Fisher Scientific, Fair Lawn, N.J.) to clarified supernatants, followed by slow stirring overnight at 4°C. The precipitated material was pelleted by centrifugation at 10,000 × g for 15 min at 4°C and resuspended in phosphate buffered saline (PBS) and 1% Nonidet P40

Substitute ([Octylphenoxy]polyethoxyethanol; USB Corp., Cleveland, Ohio) to 1/20th the original volume. The virus was purified using a 10% to 40% cesium chloride gradient and centrifugation at 26,500 rpm in an SW28 rotor (Beckman Coulter, Inc., Fullerton, Calif.) for 4 hours at 4ºC in an ultracentrifuge (Beckman Coulter, Inc.). Following centrifugation, a blue band was visualized at a density of 1.30 g/cm^3 and harvested. The recovered virus was dialyzed through a 10,000-Da-cutoff membrane (Pierce Biotechnology, Inc., Rockford, Ill.) overnight at 4°C in 4 L of PBS with stirring and multiple changes of PBS. Purified virus in PBS was aliquoted and stored at -80ºC until use. Protein concentrations were determined by a bicinchoninic acid procedure (82).

Plaque assay. The plaque assay was adapted from one previously described (Bac-N-Blue Transfection Kit; Invitrogen, Carlsbad, Calif.). Briefly, RAW 264.7 cells were seeded onto 60 mm diameter tissue culture plates at a density of 5 x 10^6 cells and allowed to adhere for 5 hours at 37ºC. Ten-fold serial dilutions of MNV-1 were made on ice using supplemented DMEM and inoculated in duplicate onto 60 mm diameter plates containing a confluent layer of RAW cells. After incubation for 1 hour at 37ºC, the inoculum was aspirated and replaced with a mixture of one part 2.5% SeaPlaque agarose and three parts supplemented DMEM, allowed to solidify and incubated at 37ºC for 24 to 48 hours until plaques were visible. For better visualization of the plaques, neutral red (0.01% final concentration) was added to 3 ml of the mixture containing 2.5% SeaPlaque agarose and supplemented DMEM, allowed to solidify, and incubated at 37ºC for 6 to 8 hours.

Multiplexed fluorescent immunoassay (MFI). Cesium chloride purified MNV-1 was covalently coupled to 5 × 10^6 carboxylated polystyrene microspheres (Luminex

Corp., Austin, Texas) at the optimal coupling concentration of 4 µg of protein per million microspheres (previously determined, data not shown) according to manufacturer's recommended protocols, except that protein coupling to the microsphere was performed in a final volume of 1 ml of 50 mM MES coupling buffer (2-[N-Morpholino]ethanesulfonic acid; Sigma-Aldrich, St. Louis, Missouri). Microspheres were stored at 4°C in the dark until use.

Testing of mouse serum samples for anti-MNV-1 antibody was done using the LiquiChip Workstation (Qiagen Inc., Valencia, Calif.). First, microspheres coupled to MNV-1 proteins were resuspended by vortexing and sonication. Multiscreen 96-well filter bottom plates (MABV N12 50; Millipore Corp., Bedford, Mass.) were pre-wetted by adding 100 µl of PBS-BSA (PBS, 1% bovine serum albumin, 0.05% sodium azide; Sigma-Aldrich) followed by removal of the liquid using a plate vacuum manifold. Approximately 2,500 microspheres in PBS-BSA and test serum at a final dilution of 1:500 in a total volume of 100 µl were added to each well. Plates were covered and incubated for 90 min on an orbital shaker at 400 rpm at room temperature in the dark. The fluid in the wells was removed using the plate vacuum manifold. Each well was washed twice by adding 100 µl of PBS-BSA, shaking at 900 rpm for 1 minute, and then removing the fluid with the vacuum manifold. The microspheres were resuspended in 100 µl of PBS-BSA and 10 µl of a 1:20 diluted $F(ab')_2$ fragment goat anti-mouse IgG (H+L) phycoerythrin conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was added to each well. The plate was covered and incubated for 90 min on an orbital shaker at 400 rpm at room temperature in the dark. Plates were washed twice as described above, and microspheres were resuspended in 100

µl of fresh PBS-BSA and analyzed on the LiquiChip Workstation that reported the median fluorescent intensity of 100 MNV-1 coated microspheres.

Receiver operating characteristic (ROC) curve. Test results of serum samples from 189 uninfected or naturally MNV-1-infected mice were used to plot a receiver operating characteristic (ROC) curve (67, 68). The ROC curve plots 1-specificity on the x-axis and the sensitivity on the y-axis which were calculated as the MNV-1 MFI threshold values varied, by using the MNV-1 IFA test result as the reference standard to detect the presence of anti-MNV-1 antibody. The area under the ROC curve represents the accuracy of the diagnostic test, with an area of 1.0 corresponding to a perfect test (100% sensitivity and 100% specificity) and an area of 0.5 representing a test with no diagnostic predictability. Based on the sensitivity and specificity calculations made to plot the ROC curve from the 189 mouse serum samples, the MFI fluorescence value corresponding to a 95% test sensitivity and the MFI fluorescence value corresponding to a 95% test specificity were used as the threshold values to discriminate a negative, intermediate or positive test result in all subsequent serum samples tested by the MNV-1 MFI.

Indirect fluorescent antibody (IFA) assay. RAW 264.7 cells in suspension culture were infected with MNV-1 as described above and incubated at 37 ºC for 24 hours, at which time there was approximately 20% CPE as indicated by the trypan blue exclusion assay. Infected cells were mixed with equal numbers of uninfected RAW cells and 10 µl of this mixture was spotted onto each spot of 12-well Teflon printed slides (Erie Scientific Co., Portsmouth, New Hampshire). Slides were acetone fixed at 4ºC for 10 minutes and stored at -80ºC until use. For evaluation of mouse serum samples, 20 µl

of test serum diluted 1:5 in PBS was added to each well and incubated at room temperature for 15 min. Slides were then washed and incubated for 15 min in the dark with 20 µl of goat anti-mouse IgG (H+L) fluorescein isothiocyanate (FITC)-labeled secondary antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) diluted 1:25 in PBS containing 1% Evan's blue dye. Evidence of specific fluorescence was evaluated using a 480 nm wavelength UV light microscope. Samples were read by three individuals experienced in evaluating IFA assay slides and classified as positive, negative or having non-specific fluorescence. If a discrepancy arose, slides were re-evaluated by the same individuals and a consensus determination was made.

Western blot analysis. Separation and transfer of cesium chloride purified MNV-1 proteins were done using the XCell SureLock Mini-Cell system (Invitrogen, Carlsbad, Calif.) according to manufacturer's recommended protocol. Proteins were separated using NuPAGE Novex 4-12% Bis-Tris Gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.). Membranes were blocked with 5% nonfat dry milk in PBS and probed with mouse serum samples diluted 1:50 in 5% nonfat dry milk in PBS for 1 hour at room temperature. Membranes were then incubated with a 1:1000 dilution of biotinylated donkey anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) in 5% nonfat dry milk in PBS for 1 hour at room temperature, and then with avidin DH and biotinylated horseradish peroxidase (Vector Laboratories, Inc., Burlingame, Calif.) for 30 minutes before development with TMB (3,3', 5,5'-tetramethylbenzidine; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was extracted from cesium chloride purified MNV-1 using the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, Calif.) according to manufacturer's recommended protocol. RNA was extracted from mouse fecal and tissue samples using the MagAttract RNA Tissue Mini M48 Kit (Qiagen Inc.) according to manufacturer's recommended protocol. Briefly, one fecal pellet or approximately 20 mg of tissue was disrupted and homogenized in Buffer RLT using 5 mm stainless steel balls and a TissueLyser (Qiagen Inc.). Fecal samples were disrupted and homogenized at 30 Hz for 10 sec and tissue samples at 20 Hz for 2 minutes twice. Fecal and tissue lysates were centrifuged for 5 min at 300 × g or for 3 min at 13000 × g, respectively, and the supernatants used for magnetic silica-based RNA purification on the BioRobot M48 Workstation (Qiagen Inc.). RNA concentration and purity was determined by measuring the A_{260} and A_{280} with a spectrophotometer.

MNV-1 specific RT-PCR primers (MNV Polymerase 5 Forward: 5'-TCTTCGCAAGACACGCCAATTTCAG-3' and MNV Polymerase 5 Reverse: 5'-GCATCACAATGTCAGGGTCAACTC-3') targeting the polymerase gene of MNV-1 (GenBank Accession AY228235) were designed using the Discover Studio Gene software package (Accelrys, San Diego, Calif.). This primer pair produces a 318-bp product from nucleotide positions 4228 to 4545 after reverse transcription and PCR amplification of MNV-1 RNA. RT-PCR was performed in duplicate according to manufacturer's recommended protocols using the OneStep RT-PCR Kit with Q-Solution (Qiagen Inc.) and 0.6 μM of each primer. Reverse transcription was done at 50ºC for 30 min followed by one cycle of 95ºC for 15 minutes to activate the DNA polymerase, 40

cycles of denaturation (94ºC, 1 min), annealing (63ºC, 1 min), and extension (72ºC, 1 min), and a final extension at 72ºC for 10 min. PCR products were evaluated on precast 3% agarose gels containing ethidium bromide (Bio-Rad Laboratories, Hercules, Calif.) and visualized with UV light.

Animals and sample collection. To evaluate anti-MNV-1 antibody production following experimental exposure to MNV-1, 4-week old female Hsd:ICR(CD-1) mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were obtained that were documented to be free of ectoparasites, endoparasites, *Mycoplasma pulmonis*, *Helicobacter* sp., known enteric and respiratory bacterial pathogens, antibodies to mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler's murine encephalomyelitis virus, ectromelia virus, polyoma virus, lymphocytic choriomeningitis virus, mouse adenovirus, mice minute virus, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, *Encephalitozoon cuniculi* and *Clostridium piliforme*. Baseline serum samples collected from the saphenous veins of all mice before experimental inoculation were negative for anti-MNV-1 antibody. Control mice were sham-inoculated with uninfected RAW 264.7 cell lysates by oral gavage (n=5) and experimental mice were inoculated with 10^7 PFU of MNV-1 infected RAW cell lysates by oral gavage (n=10). Mice were group housed by MNV-1 infection status in micro isolation cages with filter tops and provided autoclaved acidified water and standard irradiated rodent chow *ad libitum*. Fresh fecal samples were collected daily on days 0 to 7 postinoculation (PI) and blood samples were collected weekly from all mice. All manipulations, including basic husbandry, were done in a class II biological safety cabinet. At 5 weeks PI, mice were euthanized with an overdose of carbon dioxide. Blood was collected by cardiocentesis,

and the serum was separated, diluted 1:5 with PBS and stored at -20°C. The mesenteric lymph node, sections of the spleen and jejunum, and a fecal pellet from the descending colon were collected aseptically, immediately placed in liquid nitrogen and stored at -80ºC pending RNA extraction. The animal studies were approved by the University of Missouri's Animal Care and Use Committee.

To evaluate the anti-MNV-1 antibody status in mice naturally exposed to MNV-1, serum samples from various strains of immunocompetent mice from a variety of sources submitted to the University of Missouri Research Animal Diagnostic Laboratory (Columbia, Mo.) were tested by the MNV-1 MFI. To determine the threshold fluorescence values to designate a negative, intermediate or positive test result when evaluated by the MFI, sera from 121 mice positive for anti-MNV-1 antibodies by MNV-1 IFA assay and sera from 68 mice negative for anti-MNV-1 antibodies by MNV-1 IFA assay were used as reference standards. Blood was obtained by cardiocentesis from euthanized mice and the serum was collected, diluted 1:5 with PBS, and stored at -20°C.

Electron microscopy. A 200 µl aliquot of cesium chloride purified MNV-1 was diluted in PBS and centrifuged at 40,000 rpm for 1 hour in a SW41Ti rotor (Beckman Coulter, Inc.) at 5ºC to pellet the virus. The pellet was resuspended in 100 µl of chilled 1% ammonium acetate overnight at 4°C. A 10 µl drop was placed onto 300-mesh carbon-coated copper grids and allowed to settle for 10 minutes. Excess solution was wicked off and a drop of 1% uranyl acetate (aqueous) was added and stained for 10 minutes. The grids were viewed at 100 kV accelerating voltage in a JEOL 1200EX transmission electron microscope.

Statistical analysis. MFI values from mice that seroconverted in the experimentally inoculated group were compared to those of the sham-inoculated control group using the Mann-Whitney rank sum test (SigmaStat 3.1; Systat Software, Inc., Point Richmond, Calif.). A P value of less than 0.05 was considered significant. To evaluate the MNV-1 MFI, a receiver operating characteristic curve was generated using Analyse-It statistical software v1.71 (Analyse-It Software, Ltd., England) with Microsoft Excel 2003 (Microsoft Corp., Redmond, Wash).

RESULTS

MNV-1 production in suspension culture flasks. Cesium chloride purification of MNV-1 from 1-L suspension cultures of RAW 264.7 cells infected with MNV-1 yielded 3.2 mg of purified MNV-1 protein. Electron micrographs of this purified preparation indicated the sample contained MNV-1 virions with minimal contaminating material (Figure 2.1). Viral particles were approximately 30 nm in diameter, similar to that previously reported for MNV-1 (106).

Evaluation of MFI to detect anti-MNV-1 antibody. Using the MNV-1 IFA assay as the reference standard, a receiver operating characteristic curve was generated by plotting the false positive rate (1-specificity) on the x-axis vs. the true positive rate (sensitivity) on the y-axis as the threshold values of the MNV-1 MFI varied to obtain an area under the curve of 0.905 (95% confidence intervals 0.858 to 0.953), indicating the test performance is highly accurate (68). Based on the sensitivity and specificity calculated at various threshold values used to generate the curve, an MFI value of 175 corresponded to a 95% test sensitivity (69% specificity) while an MFI value of 600

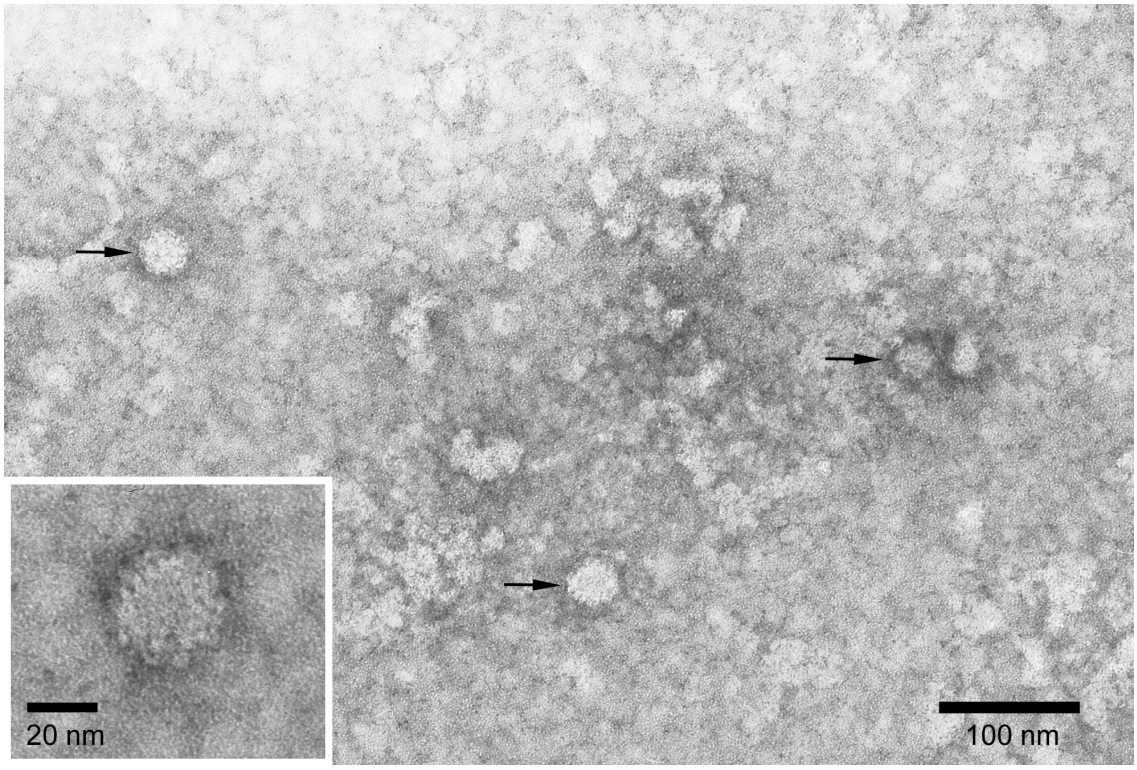

Figure 2.1. Transmission electron micrograph of negatively stained murine norovirus 1 particles (arrows) from cesium chloride-purified supernatants of infected RAW 264.7 cells grown in suspension culture.

corresponded to a 95% test specificity (39% sensitivity). To achieve maximum overall test performance, the following testing paradigm was established: sera with MFI values of < 175 were classified as negative for anti-MNV-1 antibodies, sera with MFI values from 175 to 600 were classified as intermediate and possibly containing anti-MNV-1 antibodies, and sera with MFI values > 600 were classified as positive for anti-MNV-1 antibodies. To determine the anti-MNV-1 antibody status of sera in the intermediate range (MFI values from 175 to 600), these serum samples were tested by the MNV-1 IFA assay and the outcome of this test was used to classify sera as positive or negative.

To evaluate the performance of the MNV-1 MFI and the kinetics of seroconversion of mice following oral exposure to MNV-1, sera from experimentally inoculated mice and sham-inoculated control mice were evaluated by the MNV-1 MFI and IFA assay. At 1 week PI, 2 of 10 (20%) MNV-1 inoculated mice were in the intermediate fluorescence range by the MFI. IFA assays of these two samples were positive for anti-MNV-1 antibody. The remaining eight serum samples tested negative by the MFI, but two of these samples were positive by the IFA assay. However, these two serum samples had MFI fluorescence values of 165.5 and 158.5, just under the 175 threshold value for an intermediate result. At two weeks PI and for the remainder of the study, 7 of 10 (70%) MNV-1 inoculated mice were in the intermediate or positive range by the MFI and were positive by the IFA assay. There was a steady rise in the MNV-1 MFI mean fluorescence values over time in these 7 mice (Figure 2.2) until the end of the experiment when the mean fluorescence value ± SD at 5 weeks PI was 1101.9 ± 684.6. By 5 weeks PI, five of these seven samples had reached MFI values in the positive range, while two were in the intermediate range. The remaining 3 of 10 (30%) mice orally

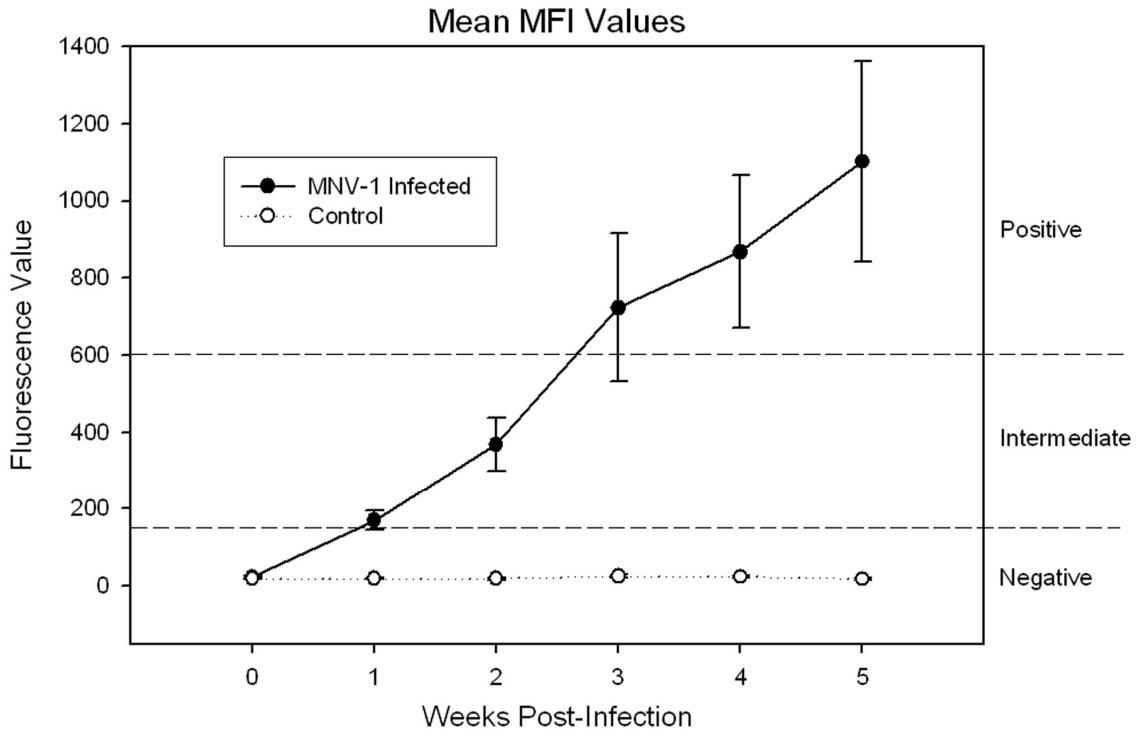

Figure 2.2. The mean MNV-1 MFI fluorescence values of sera collected weekly from mice experimentally inoculated with MNV-1 by oral gavage that seroconverted (n=7) and sham-inoculated control mice (n=5). Mice inoculated with MNV-1 showed a steady increase in MFI fluorescence values over time. An MFI fluorescence value of 175 corresponds to a 95% test sensitivity, while an MFI fluorescence value of 600 corresponds to a 95% test specificity (dashed lines). These fluorescence values were used as thresholds so that MFI fluorescence values <175 were considered negative for anti-MNV-1 antibody, values from 175 to 600 were considered intermediate and possibly containing anti-MNV-1 antibody, and values >600 were considered positive for anti-MNV-1 antibody. Error bars represent the standard errors of the mean.

inoculated with MNV-1 tested negative for anti-MNV-1 antibody by the MFI and IFA assay during the entire experiment, and had MFI fluorescence values of 12.0, 15.0 and 43.0 at week 5 PI. These three serum samples were also negative for anti-MNV-1 antibodies by Western blot assays at 5 weeks PI. Collectively, these data suggest these three mice failed to be infected with MNV-1 rather than the serologic assays failure to detect anti-MNV-1 antibody. In comparison to mice that seroconverted, serum samples from control mice inoculated with uninfected RAW cell lysates were negative by the MNV-1 IFA assay and MNV-1 MFI at all time points and had a mean MFI fluorescence value ± SD of 17.7 ± 4.1 at 5 weeks PI, which was statistically significant when compared to the values from the seven mice that seroconverted ($P < 0.05$).

Western blot analysis of the seven serum samples that contained anti-MNV-1 antibody at week 5 PI determined by the MFI and IFA assay exhibited a 59 kDa capsid protein as previously described for MNV-1 in all seven of these serum samples (Figure 2.3) (230). Western blot analyses of serum samples from control mice at 5 weeks PI were negative.

Prevalence of MNV-1 infection in research mice. To obtain an estimate of the seroprevalence of MNV-1 in research mice, mouse serum samples submitted to the Research Animal Diagnostic Laboratory at the University of Missouri (Columbia, Mo.) from research facilities in the U.S. and Canada were evaluated for anti-MNV-1 antibody by the MNV-1 MFI. Serum samples that had an MFI value > 600, corresponding to a test specificity of 95%, were classified as positive for previous infection with MNV-1. Of the 12,639 serum samples tested by the MNV-1 MFI, 2791 (22.1%) were positive for anti-MNV-1 antibody indicating widespread exposure of MNV-1 in research mouse colonies.

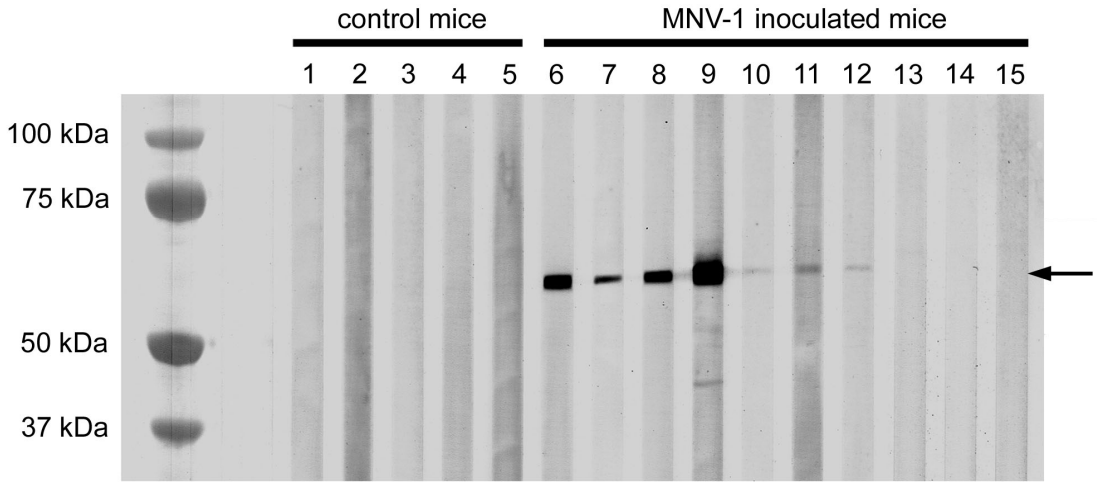

Figure 2.3. Western blot analysis of serum samples from mice 5 weeks after oral inoculation with MNV-1 infected lysates (lanes 6 to 15) or control mice sham-inoculated with uninfected RAW 264.7 lysates (lanes 1 to 5). Samples containing anti-MNV-1 antibody (lanes 6 to 12) displayed a band approximately 59 kDa in size (arrow) representing the capsid protein. Control mice and the three mice that did not seroconvert after oral inoculation with MNV-1 (lanes 13-15) did not display any specific bands by Western blot analysis.

Sensitivity and specificity of MNV-1 RT-PCR assay. To test the sensitivity of the MNV-1 RT-PCR assay, RNA was extracted from cesium chloride purified MNV-1 infected cell cultures and RNA concentrations were determined by a spectrophotometer. Log-fold serial dilutions from 100 pg to 10 ag of MNV-1 RNA were made and tested using the MNV-1 RT-PCR assay. A specific PCR product was detectable in the sample containing 100 ag of starting RNA (Figure 2.4), equivalent to a copy number of 25 virus particles assuming the purified RNA contained 100% MNV-1 RNA. The specificity of the MNV-1 RT-PCR assay was tested by using RNA extracted from uninfected RAW 264.7 cells, and fecal and tissue samples from uninfected sham-inoculated control mice. No specific or non-specific PCR products were detected from uninfected samples (data not shown). In addition, a nucleotide BLAST search of each primer did not reveal any significant nucleotide homology to non-MNV-1 sequences.

Detection of MNV-1 RNA in experimental samples. Fecal samples were collected daily for 7 days PI from mice that were given MNV-1 by oral gavage and at necropsy 5 weeks PI. MNV-1 RNA could be detected in the feces of some mice at all time points from day 0 to day 7 PI (Table 2.1), while fecal samples collected at 5 weeks PI were uniformly negative for MNV-1 RNA (Table 2.2). The three mice that were orally inoculated with MNV-1 but that did not seroconvert had detectable levels of MNV-1 RNA in their feces only for the first few days PI, most likely due to the pass through of the inoculum dose. MNV-1 RNA was detected in the mesenteric lymph node (MLN), the spleen and the jejunum of some experimentally inoculated mice by RT-PCR at 5 weeks PI (Table 2.2). All uninfected control mice samples remained negative for MNV-1 by RT-PCR throughout the duration of the study.

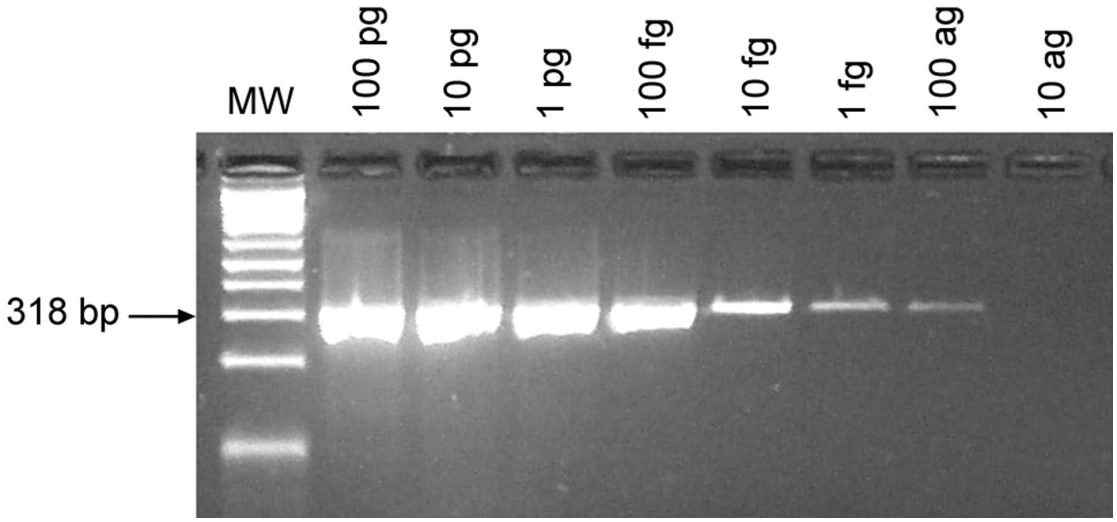

Figure 2.4. Ethidium bromide-stained agarose gel of MNV-1 RT-PCR products of log-fold serial dilutions of RNA extracted from cesium chloride-purified MNV-1-infected cell cultures. A 318-bp PCR product was generated with as little as 100 ag of RNA, representative of approximately 25 virus copies based on the estimated viral genome molecular mass of 2,366,090 Da per virion.

Table 2.1. MNV-1 detection by RT-PCR in fecal pellets collected from day 0 to day 7 postinoculation from mice inoculated with MNV-1 by oral gavage and sham-inoculated mice.

	Number positive detected by PCR							
MNV-1 status	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Uninfected (n=5)	0	0	0	0	0	0	0	0
MNV-1 inoculated (n=10)	0	10	9	7	2	4	3	2

Table 2.2. MNV-1 detection by RT-PCR in tissue and fecal samples collected at 5 weeks postinoculation from mice inoculated with MNV-1 by oral gavage and sham-inoculated mice.

	Number positive detected by PCR			
MNV-1 status	MLN[a]	Spleen	Jejunum	Feces
Uninfected (n=5)	0	0	0	0
MNV-1 inoculated (n=10)	3	1	1	0

[a]MLN, mesenteric lymph node

DISCUSSION

Murine norovirus 1 is a newly recognized pathogen of mice and is the first norovirus to be adapted to grow in cell culture (106, 230). To date, disease in mice has been described only in mice lacking components of the innate immune system, namely STAT-1 and interferon (IFN) receptors. In contrast, MNV-1 infected wild-type 129 mice do not show any clinical symptoms of disease or any tissue pathology after oral inoculation (106). High-throughput, sensitive and specific diagnostic assays are needed to identify infected mice. These assays will aid in estimating the prevalence of MNV-1 in research mouse colonies and will serve as tools for MNV-1 research studies.

The multiplexed fluorescent immunoassay was developed as a high-throughput, automated screening test over the traditional ELISA because this assay has been shown to be as sensitive if not more sensitive than ELISA (116, 192). An additional advantage of the MFI over the traditional ELISA is that up to 100 different analytes can be multiplexed in one reaction well (116) vs. an ELISA where usually only 1 analyte is screened per well. This multiplexing is the basis for the high-throughput capabilities of this diagnostic assay. Simultaneous serodetection of 10 mouse pathogens has recently been reported using this immunoassay format (115) and our laboratory currently uses this methodology to test for antibodies to 22 different mouse pathogens, including MNV-1. The MFI format also requires much less serum than an ELISA since multiple agents can be screened using one aliquot of serum. For example, only 0.2 µl of undiluted mouse serum is needed in the MFI format to test for up to 100 different agents, while single agent ELISA would require approximately 2 µl of undiluted serum per agent tested. This is a significant advantage since only a small amount of sera can be obtained from a mouse,

and allows for the capability to perform comprehensive serologic profiles from a survival blood collection from a mouse.

Based on this diagnostic approach, we were able to develop a high-throughput screening test to detect anti-MNV-1 antibodies in mice. In order to determine the MNV-1 MFI threshold fluorescence values to classify sera as positive or negative for containing anti-MNV-1 antibodies, a receiver operating characteristic curve was generated comparing the MNV-1 MFI fluorescence values to MNV-1 seroreactivity determined by the MNV-1 IFA test (67, 68). This curve is a plot of the false positive rate (1-specificity) on the x-axis and the true positive rate (sensitivity) on the y-axis which are calculated as the threshold values vary. The area under the curve represents the accuracy of the diagnostic test with an area of 1.0 corresponding to a perfect test (100% sensitivity and 100% specificity) while an area of 0.5 represents a test with no diagnostic predictability. The area under the curve for the MNV-1 MFI was 0.905 indicating an excellent or very accurate test. Calculating the sensitivity and specificity of the MNV-1 MFI at various fluorescence threshold values indicated the assay was 95% sensitive at a threshold value of 175 and was 95% specific at a threshold value of 600. To achieve maximum test performance, a testing paradigm was developed similar to one proposed previously (67). MNV-1 MFI fluorescence values less than 175 were classified as negative for anti-MNV-1 antibody, while MFI values greater than 600 were classified as positive for anti-MNV-1 antibody. Sera with MFI values from 175 to 600 were classified as intermediate and possibly containing anti-MNV-1 antibodies. To discern the MNV-1 reactivity of these intermediate sera, they were further tested by the MNV-1 IFA assay and the result of this test was used for final classification as positive or negative. This testing paradigm has

the advantage of providing high-throughput results with a 95% sensitivity or a 95% specificity for fluorescence values below 175 or above 600, respectively, using just the MNV-1 MFI, while using the more labor-intensive IFA assay to characterize the intermediate MFI fluorescence values. Sera from naturally infected research mice were used to develop the threshold values of this testing paradigm since this population is more representative of the target test population than mice from experimental infection (67). As with any diagnostic assay, there is a possibility of false positive reactions, such as from cross-reactivity of the MNV-1 MFI with antibodies to other mouse pathogens. However, as part of the validation of the MNV-1 MFI, serum samples from mice infected with other known murine pathogens were tested and were consistently negative (data not shown).

In mice experimentally infected with MNV-1, the sensitivity and specificity for detecting anti-MNV-1 antibody based on this testing paradigm were both 100% in mice at 5 weeks PI. Seven of ten (70%) mice orally inoculated with MNV-1 were correctly identified as positive samples containing anti-MNV-1 antibody by either a positive MFI fluorescence value (which was also confirmed by IFA), or an intermediate MFI fluorescence followed by a positive IFA test. In contrast, the remaining three samples from experimentally inoculated mice plus the five control mice which did not contain anti-MNV-1 antibody were correctly classified as negative by both the MFI and IFA assay. These mice were negative by all testing formats including the MFI, IFA assay, Western blot assay, and PCR of feces and tissues, providing additional support that they did not seroconvert or have any virus present beyond the inoculum dose. We speculate that the three mice failed to seroconvert after oral inoculation with MNV-1 because the

MNV-1 used for inoculation was a tissue culture adapted strain and the infectivity was possibly reduced. In support of this explanation, the original virus was a third passage plaque-purified strain of MNV-1 that was previously reported to have an attenuated virulence *in vivo* after serial passage in cell culture (230). Alternatively, the infection simply was not successful after oral inoculation, with the dose, dose frequency, route of inoculation, or immune status of the mouse all potentially playing a role in preventing development of an antibody response. Challenge studies with human volunteers likewise display an incomplete seroconversion rate of all subjects after experimental norovirus infection (100, 126, 127).

A large number of mouse serum samples submitted to the Research Animal Diagnostic Laboratory at the University of Missouri (Columbia, Mo.) from research institutions throughout the United States and Canada were screened by MFI for anti-MNV-1 antibody to estimate the prevalence of MNV-1 infection in research mice. Data collected revealed that 2791 (22.1%) of 12,639 serum samples were positive for anti-MNV-1 antibody, indicating that MNV-1 infection is widespread in research mouse populations. A previous report of infectious agents in mice by our diagnostic laboratory identified helicobacter as the most prevalent pathogen in mice with 32% of all mouse fecal samples testing positive by PCR, while mouse parvovirus (MPV) was the most prevalent viral pathogen in mice with approximately 2.5% of serum samples containing anti-MPV antibodies (129). Therefore, MNV-1 is among the most prevalent pathogens, and likely the most prevalent known viral pathogen, in mice used for research. It is not surprising that MNV-1 infection rates are so high since this viral pathogen is newly recognized, clinical signs cannot be used to determine infection status, and practices for

exclusion or elimination of this pathogen from research mouse colonies by testing and control measures are only beginning.

In experimental mice orally inoculated with MNV-1, fecal shedding and persistence of the virus in tissues was variable. MNV-1 RNA was detected in the feces by RT-PCR out to day 7 PI in 2 of 10 mice orally gavaged with MNV-1. However, as feces were not evaluated again for virus until 5 weeks PI, at which point all fecal samples were negative for MNV-1, the endpoint of fecal shedding of MNV-1 was not determined. This duration of viral shedding in the feces of mice is not inconsistent with fecal shedding data in human norovirus infections as people may shed virus for up to 2 weeks PI (57, 173). MNV-1 RNA was detected in the mesenteric lymph node, spleen and jejunum in some mice even after 5 weeks PI. These data are in contrast to results obtained previously in which 129 wild-type mice infected orally with MNV-1 did not have any detectable viral RNA by day 3 PI in the feces, spleen or intestines (106). Two reasons could account for these differences: (i) the immunologic responses to MNV-1 may be different in outbred ICR mice compared with inbred 129 mice or (ii) we used a higher viral inoculation dose of 1×10^7 PFU per mouse while the previous study inoculated mice with 3×10^4 PFU. Although the mechanisms of MNV-1 persistence in tissues are unknown, further studies are planned to evaluate the different immunologic responses from various stocks and strains of mice to MNV-1, the duration of fecal shedding from these various mice, and the presence of virus in different tissues at multiple time points postinoculation.

The MNV-1 seroreactivity data reported here suggests that murine norovirus 1, a recently discovered pathogen in mice, is present in a high percentage of contemporary

research mice. Currently, only limited information is known about the effects that MNV-1 has on the mouse immune system and whether these effects, or any other yet to be discovered effects, have on research. To assist further research on MNV-1, we have developed a MNV-1 microsphere-based multiplexed fluorescent immunoassay to use as a high-throughput, sensitive and specific, serologic assay. In addition, we have validated a MNV-1 IFA and Western blot assay to be used as confirmatory tests for the MFI screening assay. Finally, we have developed a very sensitive and specific RT-PCR primer set to detect MNV-1 in tissues and feces. These assays will provide the diagnostic tools necessary to aid in the detection of MNV-1 infection in mice, enabling further research studies with MNV-1 and facilitating establishment of laboratory or research mouse colonies free of MNV-1.

CHAPTER III

Persistent Infection with and Serologic Cross-Reactivity of Three Novel Murine Noroviruses

INTRODUCTION

Murine norovirus 1 (MNV-1) is a recently discovered pathogen that infects laboratory mice (106). Mice lacking specific components of the innate immune system, such as signal transducer and activator of transcription 1 or the interferon αβ and γ receptors, succumb to lethal infection after inoculation with MNV-1, whereas other immunocompromised strains, such as recombination-activating gene knockout mice, and wild-type mice survive after inoculation (84, 106). A recent report by our laboratory found that 22.1% of 12,639 mouse serum samples from various research institutions in North America contained antibodies to MNV-1, thus making MNV-1 the most prevalent viral pathogen infecting research mice (84, 129). Previous studies showed that after oral inoculation, MNV-1 was undetectable in the feces of strain 129 mice or was shed briefly (7 days) in the feces of a small percentage of ICR mice (84, 106). Persistence of MNV-1 in tissues of immunocompetent mice after experimental inoculation is variable, with 1

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report (106) demonstrating clearance of detectable virus by day 3 postinoculation (PI), whereas another (84) suggests more prolonged tissue persistence (to 5 weeks PI) in a small percentage of inoculated mice.

In this report, we describe the isolation of 3 novel, genetically distinct MNV strains and compare the serologic cross-reactivity of these new strains with each other and MNV-1. We also report evidence for persistent infection with and prolonged fecal shedding of these 3 novel MNV strains in immunocompetent mice.

MATERIALS AND METHODS

Isolation of novel murine noroviruses. Mesenteric lymph nodes (MLN) were aseptically collected from mice that were clinically normal, euthanized by overdose of inhaled carbon dioxide, and positive for antibodies to MNV by a multiplex fluorescent immunoassay previously described (84). These mice were from geographically different regions of the United States and comprised both males and females from 7-week-old to adult of various stocks and strains, such as Swiss Webster, DBA/2, and SJL mice. MLN from mice were pooled by geographic location in groups of 15 to 20 in 4 mL of Dulbecco's modified Eagle's medium(HyClone, Logan, Utah) supplemented with 10% low-endotoxin fetal bovine serum (Cambrex, East Rutherford, N.J.), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10 μg/ml of ciprofloxacin (supplemented growth medium). Pooled samples were processed in a blender (Stomacher 80 Lab Blender, Tekmar Company, Cincinnati, Ohio) and clarified by centrifugation for 10 min at 10,000 × g. RAW 264.7 cells were grown to 80% confluence in 25 cm^2 cell culture flasks and 1 mL clarified MLN homogenate was

inoculated onto RAW cells and incubated for 1 hour at 37°C and 5% CO_2, after which the inoculum was replaced with fresh supplemented growth medium and incubated at 37°C with 5% CO_2. Cultures were monitored visually, and those with cells displaying cytopathic effect (CPE), suggestive of MNV infection, were passaged and the virus was plaque purified. Propagation, concentration, purification and plaque assay of all MNV isolates were performed as previously described (84). The MNV-1 strain was a kind gift from Herbert W. Virgin (Washington University School of Medicine, St. Louis, Mo).

Nucleotide sequencing. RNA was extracted by use of QIAamp Viral RNA Mini Kits (Qiagen, Valencia, Calif.) according to manufacturer's recommended protocol from MNV samples propagated in vitro. Reverse transcriptase–polymerase chain reaction (RT-PCR) assays were done by means of the OneStep RT-PCR Kit (Qiagen) and 0.6 μM of each primer, according to manufacturer's recommended protocol. To obtain the nucleotide sequence of the putative novel MNV strains, RT-PCR was first performed with 9 overlapping primer pairs, each producing amplicons 800 to 900 base pairs in size and collectively spanning the entire genome (excluding the termini). Primers were designed using the Discover Studio Gene software package (Accelrys, San Diego, Calif.) to MNV-1 nucleotide sequence (GenBank accession no., AY228235). Successful RT-PCR products were purified either by using the Montage PCR Centrifugal Filter Devices (Millipore Corp., Bedford, Mass.) or by separation on a 1.5% SeaPlaque GTG Agarose Gel (Cambrex Corp., Rockland, Maine) followed by purification using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp., Orange, Calif.). Nucleotide sequencing of purified PCR products was performed in both directions by SeqWright DNA Technology Services (Houston, Texas). Fragments corresponding to gaps in the

nucleotide sequences of the novel MNV isolates were amplified and sequenced by use of specific primer pairs designed from flanking sequence data, and the end sequences were obtained by use of 3' and 5' RACE Systems (Invitrogen, Carlsbad, Calif.). Contig assembly was performed with Sequencher Software (Gene Codes Corp., Ann Arbor, Mich.).

Experimental infection studies. For experimental infection with MNV strains, groups of 4-week-old female Hsd:ICR(CD-1) mice (Harlan Sprague-Dawley, Indianapolis, Ind.) were obtained that were free of ectoparasites, endoparasites, *Mycoplasma pulmonis*, *Helicobacter* sp., known enteric and respiratory bacterial pathogens, and antibodies to mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler's 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, *Encephalitozoon cuniculi* and *Clostridium piliforme*. Serum and fecal samples were collected from all mice on day 0 and were negative by serologic and RT-PCR testing, respectively, for MNV infection. Each group of experimental mice (n=10) was inoculated by oral gavage with 1×10^6 plaque forming units of an MNV strain in RAW 264.7 cell lysates clarified by centrifugation at 3000 × g for 10 min. Control mice (n=10) were sham-inoculated with clarified uninfected RAW 264.7 cell lysates. Mice were group-housed in static filter top cages according to MNV strain and were provided autoclaved, acidified water and standard irradiated rodent chow *ad libitum*. All mouse manipulations were performed in a class II biological safety cabinet.

Fresh fecal samples were collected every other day from day 2 to 14 PI (except for day 6), and weekly thereafter for some groups until 8 weeks PI. At 8 weeks PI, mice were euthanized with an inhaled overdose of carbon dioxide. MLN, sections of the spleen and jejunum, and a fecal pellet from the descending colon were collected aseptically, immediately placed on ice, and stored at -80ºC pending RNA extraction. Blood was collected by cardiocentesis, and the serum was separated, diluted 1:5 with phosphate-buffered saline, and stored at -20°C. All animal studies were approved by the University of Missouri's Animal Care and Use Committee.

Microsphere fluorescent immunoassay (MFI). Virus particles from each MNV strain were propagated, purified by cesium chloride gradient centrifugation and attached to a unique carboxylated microsphere (Luminex Corp., Austin, Texas) at 4 µg protein per 1×10^6 microspheres, as previously described (84). Evaluation of mouse serum samples for anti-MNV antibody was conducted by indirect fluorescent antibody (IFA) assay and MFI as previously described, with a few modifications to the MFI (84). Modifications included using approximately 1250 microspheres per well, 1 hour incubation times with the primary and secondary antibodies, and the addition of 10 µL PBS containing 0.02% TWEEN 20, 0.05% sodium azide, and 2% formalin (Sigma-Aldrich, St. Louis, Missouri) per well as a stop solution after completion of the final washing step. Serum samples collected at 8 weeks PI from mice experimentally infected with a single strain of MNV were tested by MFI against homologous antigen (same MNV strain) and heterologous antigens (other MNV strains). Serum samples from sham-inoculated control mice at 8 weeks PI were tested for anti-MNV antibodies by MFI using all MNV antigens.

RT-PCR. In light of the nucleotide sequences of the 4 MNV strains, a RT-PCR primer pair (Forward: 5'-CAGATCACATGCTTCCCAC- 3', Reverse: 5'-AGACCACAAAAGACTCATCAC-3') was designed to bind within conserved regions of the MNV genome by use of the Genetics Computer Group Wisconsin Package (Genetics Computer Group, Inc., Madison, Wisc.), and the sensitivity of the MNV RT-PCR was assessed as previously described (84). RNA was extracted from mouse fecal and tissue samples from experimental mice by use of the MagAttract RNA Tissue Mini M48 Kit (Qiagen, Valencia, Calif.) as previously described (84). RT-PCR was performed in duplicate using the OneStep RT-PCR Kit with Q-Solution (Qiagen, Valencia, Calif.) according to manufacturer's recommended protocol and thermocycling parameters consisting of reverse transcription at 50ºC for 30 min; activation of DNA polymerase at 95ºC for 15 minutes; 40 cycles of denaturation (94ºC, 30 sec), annealing (60ºC, 30 sec), and extension (72ºC, 30 sec); and a final extension at 72ºC for 10 min. RT-PCR products were separated on precast 3% agarose gels containing ethidium bromide (Bio-Rad Laboratories, Hercules, Calif.) and visualized under ultraviolet light.

Statistical analysis. We performed a two-way analysis of variance and the Holm–Sidak pairwise multiple comparison procedure by using SigmaStat 3.1 software (Systat Software, Point Richmond, CA) on MFI values obtained at week 8 PI. A P value of less than 0.05 was considered statistically significant.

RESULTS

Isolation of 3 novel MNV strains. To obtain putative MNV strains infecting laboratory mice, MLN samples from mice serologically positive for MNV were

processed and inoculated onto permissive cell cultures. Three novel MNV strains were successfully isolated from 3 colonies of research mice from different geographical regions of the United States. When cell cultures were infected with each MNV strain at a multiplicity of infection of 0.1, the MNV-3 and MNV-4 strains had similar growth kinetics to MNV-1, producing 100% CPE within 48 hours, whereas MNV-2 required approximately 72 hours to reach 100% CPE. Nucleotide identities between MNV strains ranged from 86% to 91% (data not shown) indicating that all strains were genetically distinct. The 3 new strains were named MNV-2, MNV-3 and MNV-4 (GenBank accessions DQ223041, DQ223042 and DQ223043).

Anti-MNV antibody production and MFI. To assess the serologic cross-reactivity between the different MNV strains, we experimentally inoculated groups of 10 ICR mice with each MNV strain. No mice developed any clinical disease after oral inoculation with any of the MNV strains. At 8 weeks after oral inoculation with MNV, serum samples were collected and tested by IFA assays that used MNV-1-infected cells as antigen. Only 3 of 10 mice inoculated with MNV-1 seroconverted, according to the MNV-1 IFA assay. In contrast, all mice inoculated with MNV-2, MNV-3, and MNV-4 tested positive for anti-MNV antibodies on the MNV-1 IFA assay. Sham-inoculated control mice and all day 0 serum samples were negative for anti-MNV antibody by the IFA assay. We also tested all mouse serum samples obtained at week 8 PI by MFI using each MNV strain individually as the antigen. The fluorescence values (mean ± standard error) for each group of mice were calculated from mice that seroconverted (Figure 3.1). A threshold fluorescence value of 600 was used as the cutoff to determine a positive

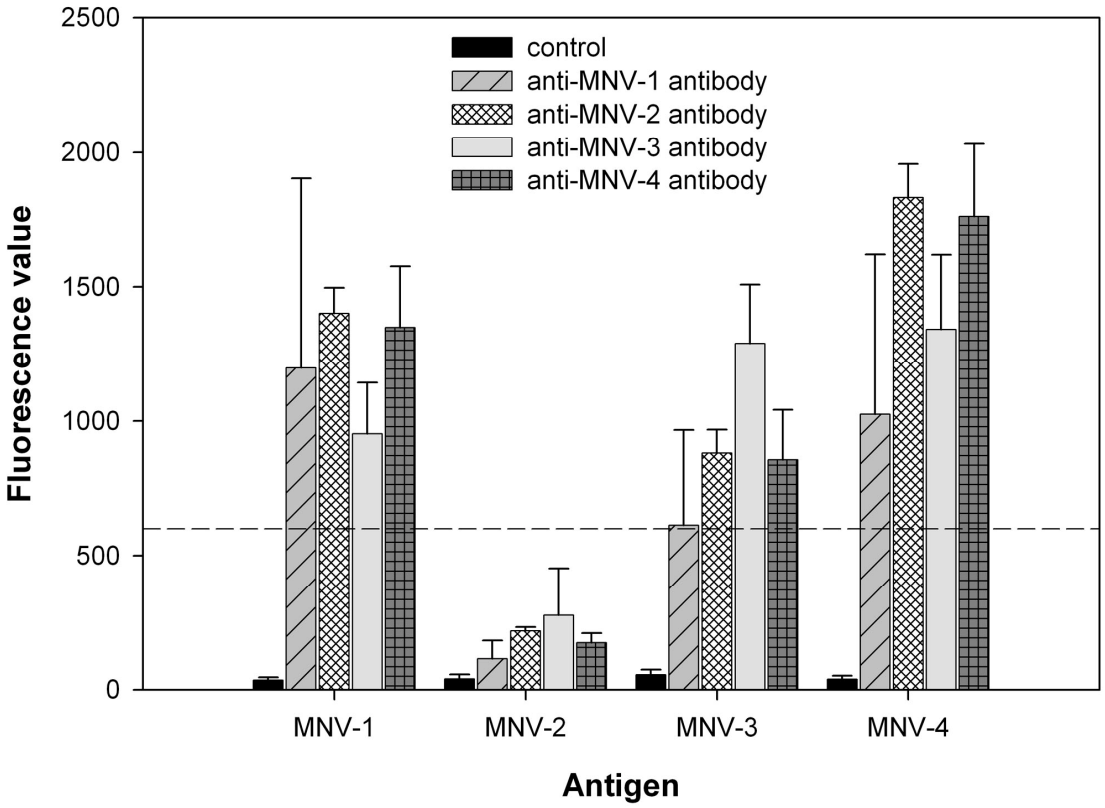

Figure 3.1. Serum antibody responses of mice that were positive for anti-MNV antibodies by the MNV indirect fluorescent antibody assay after oral inoculation with MNV-1 (n = 3), MNV-2 (n = 10), MNV-3 (n = 10), or MNV-4 (n = 10) and of sham-inoculated control mice (n = 10). Serum samples were tested in microsphere fluorescent immunoassays using MNV-1, MNV-2, MNV-3, or MNV-4 as antigen. Bar height represents the mean fluorescence value, and error bars represent the standard error of the mean. A fluorescence value of 600 (dashed line) was used as the threshold for a positive result.

result, as previously described (84). The MNV-1, MNV-3 and MNV-4 antigens displayed good serologic cross-reactivity with sera from MNV-1, MNV-2, MNV-3 and MNV-4-inoculated mice (Figure 3.1). When MNV-2 was used as antigen for the MFI, mean fluorescence values were low for all 4 groups of serum samples containing anti-MNV antibody (Figure 3.1), including serum samples containing the homologous anti-MNV-2 antibody.

To compare the performance of the 4 MNV antigens statistically, a two-way analysis of variance test and the Holm-Sidak pairwise multiple comparison procedure were performed on MFI data generated from all mouse serum-antigen combinations. No statistically significant differences were found in pairwise comparisons between MNV-1 and MNV-3 as well as MNV-1 and MNV-4. All other pairwise comparisons between MNV antigens yielded statistically significant ($P < 0.05$) differences.

RT-PCR of fecal and tissue samples. On the basis of the nucleotide sequences of the 4 MNV strains, we designed a RT-PCR primer pair that bound within conserved regions of the MNV genome and subsequently used these primers for the detection of MNV in mouse fecal and tissue samples. This primer pair produced a 187-basepair product from nucleotide positions 5473 to 5659 which is located in the capsid gene coding region of the MNV consensus sequence, and exhibited a sensitivity of approximately 25 virus copies when tested with RNA extracted from cesium chloride-purified virus (data not shown).

To determine the endpoint of fecal shedding in mice experimentally inoculated with MNV, we collected fecal samples every other day from day 2 to day 14 PI (with the exception of day 6) and weekly thereafter for most groups of mice until 8 weeks PI. Only

60% of mice inoculated with MNV-1 were fecal-positive for MNV by RT-PCR at day 2 PI, after which there was no detectable MNV in their feces at any other evaluated time point to day 14 PI (Table 3.1). Because MNV-1 fecal shedding had stopped, fecal samples were not evaluated from MNV-1 inoculated mice after day 14 PI. In contrast, all mice inoculated with the other 3 strains had detectable levels of MNV in the feces from day 2 to day 14 PI and from week 3 PI to week 8 PI, with the exception of one fecal sample at week 5 PI from a single mouse inoculated with MNV-4 (Table 3.1).

To evaluate whether MNV persistently infects tissues in mice after experimental inoculation, we collected samples of MLN, jejunum and spleen at 8 weeks PI and tested them by MNV RT-PCR (Table 3.2). All tissue samples from mice inoculated with MNV-1 were negative for virus at 8 weeks PI. In mice inoculated with MNV-2, MNV-3, or MNV-4, all MLN and jejunum samples were positive for virus, with the exception of 1 jejunum sample from a mouse inoculated with MNV-4. MNV was detected in the spleen at 8 weeks PI in 60%, 100%, and 40% of mice inoculated with MNV-2, MNV-3, and MNV-4, respectively.

DISCUSSION

Herein we report the isolation of 3 new murine norovirus strains—MNV-2, MNV-3 and MNV-4—from mesenteric lymph nodes of MNV-seropositive mice obtained from various geographical locations in the United States. Pairwise comparison of nucleotide identities of the entire genomes confirmed that all the MNV strains are genetically different from one another. This finding is not surprising, as human

Table 3.1. Serial detection of MNV by RT-PCR of fecal samples from mice at various time points after sham inoculation or oral inoculation with MNV-1, MNV-2, MNV-3, or MNV-4.

Infection group	Number of animals positive for virus (10 mice inoculated per group)													
	Day								Week					
	0	2	4	6	8	10	12	14	3	4	5	6	7	8
Uninfected	0	0	0	nd	0	0	0	0	nd	nd	0	0	0	0
MNV-1	0	6	0	nd	0	0	0	0	nd	nd	nd	nd	nd	nd
MNV-2	0	10	10	nd	10	10	10	10	nd	nd	10	10	10	10
MNV-3	0	10	10	10	10	10	10	10	10	10	10	10	10	10
MNV-4	0	10	10	nd	10	10	10	10	nd	nd	9	10	10	10

nd, not done

Table 3.2. Detection of MNV by RT-PCR of tissue samples from mice 8 weeks after sham inoculation or oral inoculation with MNV-1, MNV-2, MNV-3, or MNV-4.

	Number positive (10 mice inoculated per group)		
Infection group	Mesenteric lymph node	Jejunum	Spleen
Uninfected	0[a]	0	0
MNV-1	0	0	0[b]
MNV-2	10	10	6
MNV-3	10	10	10
MNV-4	10	9	4

[a]n = 9; 1 sample was inadvertently lost during processing

[b]n = 6; 4 samples were inadvertently lost during processing

noroviruses exhibit great sequence diversity, showing as little as 51.1% nucleotide identity between strains in different genogroups (108). Factors contributing to the sequence diversity among MNV strains may include genomic recombination of RNA viruses already reported to occur in human noroviruses (5) and a high mutation rate due to the lack of a 3'-to-5' exonuclease proofreading activity of RNA-dependent RNA polymerases (43, 232).

We used the MFI to evaluate the antigenicity of all 4 MNV strains by using sera from experimentally inoculated mice containing anti-MNV antibodies to each MNV strain. The MNV-1, MNV-3, and MNV-4 viral antigen preparations performed similarly and displayed strong serologic cross-reactivity with antibodies against all 4 MNV strains. However, the MNV-2 antigen preparation produced low mean fluorescence values when tested with this same set of positive serum samples, including sera containing the homologous anti-MNV-2 antibody. Plaque titrations of the cesium chloride purified MNV antigen preparations used in the MFI revealed that the viral titer of the MNV-2 preparation was 2 log lower than those of the other 3 MNV strains (data not shown). Therefore, although the same amount of protein was used for each MNV antigen preparation in the MFI, the poor performance of the MNV-2 antigen preparation most likely was due to markedly less virus in the antigen preparation, and not to poor antigenicity or serologic cross-reactivity. In support of this hypothesis, the MFI fluorescence values of positive serum samples, although low, were still greater than those from sham-inoculated control mice, and reactivity of the MNV-2 antigen with sera from MNV-2-inoculated mice was similar to that of sera containing antibodies against the other 3 MNV strains (Figure 3.1). Subjectively, the MNV-4 antigen preparation

produced the greatest overall reactivity with sera from MNV-infected mice. Therefore, of the antigen preparations evaluated, we propose the use of the MNV-4 antigen preparation in MNV MFI.

A previous studies in immunocompetent mice experimentally inoculated with MNV-1 demonstrated viral fecal shedding for only 7 days after inoculation and little evidence of persistent infection, because only a small percentage of mice demonstrated virus in tissues at 5 wk PI (84). In the present study, mice experimentally inoculated with MNV-1 likewise demonstrated brief viral fecal shedding and no evidence of tissue persistence, because only 60% of mice had detectable levels of MNV RNA in the feces at day 2 PI and no detectable virus in the feces at later time points or in tissue samples at 8 weeks PI. In contrast, all 30 mice inoculated with MNV-2, MNV-3 or MNV-4 had detectable virus in the MLN at 8 weeks PI and in the feces from day 2 to week 8 PI, with the exception of 1 fecal sample at week 5 PI. This result indicates that mice can become persistently infected with MNV and can persistently shed virus in the feces after experimental inoculation with these newly isolated MNV strains. Prolonged fecal shedding of MNV-2, MNV-3 and MNV-4 may explain, in part, the high serologic prevalence of MNV in research mice previously reported (84). We suspect that the disparity in duration of fecal shedding and tissue persistence between MNV-1 and the 3 novel MNV strains may be because the MNV-1 used in the present study is a tissue-culture adapted strain that has been reported to have a reduced virulence in vivo (230), whereas MNV-2, MNV-3 and MNV-4 have been passaged minimally in vitro and may represent field isolates that have not undergone attenuation in culture.

The serologic and RT-PCR assays we report provide necessary tools to diagnose infections with all currently known MNV strains in research mice. The MFI is a relatively inexpensive, high-throughput assay that is well suited for screening populations of mice for MNV exposure. The MNV RT-PCR assay can be used to test mouse feces for active viral shedding or MLN for the presence of MNV-specific RNA. Because all 3 novel MNV strains cause persistent infections and prolonged fecal shedding in mice to at least 8 weeks PI, there is a large window of time in which the MNV RT-PCR assay can be used to detect MNV infection and shedding in mice. We did not determine the end point of fecal shedding and tissue persistence of the novel MNV strains we did not collect samples beyond 8 weeks PI. However, evaluation of samples collected from 4- to 6-month-old mice from an endemically infected breeding colony indicated that 95% of fecal samples and 90% of MLN samples were positive for MNV by RT-PCR (data not shown), suggesting that fecal shedding and tissue persistence continues beyond 8 weeks PI..

In conclusion, we have isolated 3 novel murine noroviruses—MNV-2, MNV-3 and MNV-4—and developed immunologic and molecular assays capable of detecting these strains. All 4 MNV strains were serologically cross-reactive, and the MNV-1, MNV-3, and MNV-4 antigens displayed robust reactivity in the MFI. We also developed a primer set from conserved regions of the viral genomes that is able to detect all 4 known strains of MNV. Finally, we used RT-PCR to document that mice experimentally infected with the novel MNV-2, MNV-3 and MNV-4 strains persistently shed virus in the feces for the duration of the 8 week study and that virus was present in the tissues at 8 weeks PI, suggesting that MNV can cause persistent infections in mice.

CHAPTER IV

Molecular Characterization of Three Novel Murine Noroviruses

INTRODUCTION

Noroviruses are a group of genetically and antigenically diverse viruses that infect both humans and animals (48, 106, 108). In humans, norovirus infections occur worldwide and are responsible for as much as 94% of outbreaks of acute nonbacterial gastroenteritis (45, 46, 63, 121). The disease in humans is typically mild and self-limiting with symptoms of nausea, fever, vomiting, and diarrhea lasting for 24 to 48 hours (63). Transmission of noroviruses takes place by ingesting fecally contaminated food or water sources, having direct contact with infected individuals or surfaces, or being exposed to infected aerosolized vomitus (17, 135, 142). Outbreaks of acute nonbacterial gastroenteritis caused by noroviruses have been reported in nursing homes, hospitals, schools, cruise ships, restaurants, and naval ships where close contact aids in the spread of the virus (17, 47, 93, 135). Noroviruses are also quite stable in the environment, able to cause infection and clinical illness in people even after attempts to inactivate the virus with chlorine, ether, acid, or heat treatments (36, 114).

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Noroviruses are single-stranded, positive-sense RNA viruses within the family *Caliciviridae*. The norovirus genome is approximately 7.3 to 7.8 kb, polyadenylated, and contains 3 open reading frames (ORF) (63). The first ORF (ORF1) encodes a nonstructural polyprotein that is proteolytically cleaved to produce an NTPase, 3C-like proteinase, and RNA-dependent RNA polymerase. The second ORF (ORF2) encodes the major capsid protein (VP1), while the third ORF (ORF3) encodes a small basic structural protein (VP2) important for the enhanced expression and stability of the capsid protein (12). The capsid protein of ORF2 can be further subdivided into two domains, the N-terminal S domain which forms the icosahedral shell of the capsid and is more conserved among caliciviruses, and the C-terminal P domain which forms the protruding arm of the capsid protein, is more variable in sequence, and is thought to contribute to strain specificity (188).

The cloning and sequencing of Norwalk virus, the prototype strain of noroviruses, allowed for the development of molecular techniques to characterize and compare norovirus strains (140, 236). These molecular assays have vastly improved the knowledge of the taxonomic classifications, genetic diversity, diagnostic capabilities, and epidemiologic study of norovirus outbreaks in humans (17, 142, 172). Noroviruses have been divided into five genogroups based on comparisons and classification of their nucleotide sequences or predicted amino acid sequences of the polymerase or capsid open reading frames (6, 106, 108, 174). The majority of noroviruses that cause outbreaks of enteric disease in humans are classified into genogroups I and II while animal noroviruses are placed in genogroups II, III and V (106, 108, 174, 221).

Recently, a murine norovirus (MNV) was reported to cause lethal infection in a colony of immunodeficient mice and was designated as a member of a new genogroup (genogroup V) based on comparisons of the amino acid sequence of the capsid gene (106). This newly described pathogen of mice, murine norovirus 1 (MNV-1), can be grown in cell culture, providing the first report of *in vitro* cultivation of a norovirus (230). Our laboratory has developed serologic assays to detect antibodies against MNV in the sera of mice (84, 85). We used these assays to evaluate 12,639 serum samples from laboratory mice from multiple research institutions in North America and found that 22.1% of serum samples contained antibodies to MNV, making MNV the most prevalent viral pathogen infecting research mice (84). Based on the genetic diversity of human norovirus strains circulating in people and the high mutability of RNA viruses, we hypothesized that substantial genetic diversity exists among MNV strains infecting laboratory mice. Tissue samples were collected from geographically distinct populations of laboratory mice with serologic evidence of infection and used for virus isolation (85). Our laboratory isolated three novel MNV strains designated MNV-2, MNV-3 and MNV-4 (85). Interestingly, these three novel MNV strains demonstrated dramatic differences to MNV-1 in pathogenicity since they produce persistent tissue infections in experimentally inoculated immunocompetent mice while infection with MNV-1 produces only a transient infection typically cleared within the first week postinoculation (84, 85, 106). In addition, the three novel MNV strains were shed in the feces by all experimentally inoculated mice for the duration of the 8 week study while MNV-1 was typically shed in the feces of mice for less than 1 week after experimental inoculation (84, 85, 106).

In this report, we describe the molecular characterization of three novel MNV strains, MNV-2, MNV-3 and MNV-4, to verify their genetic classification as murine noroviruses in light of their differences in pathogenicity to MNV-1, and to demonstrate that MNV-2, MNV-3 and MNV-4 are indeed genetically distinct from MNV-1, supporting their classification as novel MNV strains.

MATERIALS AND METHODS

Isolation, propagation and purification of MNV-2, MNV-3 and MNV-4. The MNV-1 strain was a kind gift from Herbert W. Virgin, Washington University School of Medicine (St. Louis, Mo). The isolation, propagation and purification of MNV-2, MNV-3 and MNV-4 were described previously (85).

Reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleotide sequencing. To determine the entire genomic sequences of the three novel murine norovirus isolates, RT-PCR and nucleotide sequencing were performed. A set of nine oligonucleotide primer pairs producing overlapping amplicons 800 to 900 base pairs in length were designed using the Discover Studio Gene software package (Accelrys, San Diego, CA) based on the nucleotide sequence of MNV-1 (GenBank Accession AY228235) (Table 4.1). These primer pairs spanned from nucleotide position 7 to 7148 of the 7.3 kilobase MNV-1 genome. RNA was extracted by use of QIAamp Viral RNA Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's recommended protocol from cell cultures infected with putative novel MNV strains that had been plaque purified. RNA samples were initially subjected to RT-PCR using the nine primer pairs at a concentration of 0.6 μM for each primer. RT-PCR was performed using the

Table 4.1. Primers used to amplify and sequence novel murine noroviruses.

MNV strain	Primer sequence (5' to 3')		Amplicon size	Location (nt)
	Forward	Reverse		
MNV-1	TCTAGAAGGCAACGCCATC	TGTTGAGAATGTTGAGCGG	885	7 to 891
	CCGGCCAATTAAGAACCTAC	AGCATCTGAAGCCTCAGTG	879	839 to 1717
	ACCACTGGGATGCTTACAAGGG	TGCAGAATGGACGTGATGACG	844	1624 to 2467
	CAGAAGGATAAAGGAGGCCC	AGGCTCTCTTGACAAGGAC	863	2396 to 3258
	ATGGTCCTGGAGAATGGGTG	TCCCGTAGATCTTGTCTGGC	880	3198 to 4077
	GAGATGGGTAAATCCATGCG	ATTAGTTGGGAGGGTCTCTG	869	3999 to 4867
	CCAGCTCCTCTGGACTAAAG	CAGAGACCACAAAAGACTCATC	879	4784 to 5662
	ATGCTACCCAGGATCAAGAG	TCGATCTGACGCATGTAGG	811	5564 to 6374
	TACAACGATGGGCTACTGG	GGATGTCACTTGCCTACCTG	872	6277 to 7148
MNV-2	TGCCAGGTGATCTACAACGG	ATCTTGATGGCAGCTTGCG	1028	2286 to 3313
	TGGTTGTTGCCCTTGTACCAC	TGACAACCTCAAAGAAGCCGTC	1191	5420 to 6610
	ACTCAAGGCACCTACACGAAC	AATGCATCTAAATACTACT	140	7242 to 7381
MNV-3	CGTTGGCATGACTGTCCCTTAC	GCCCAATGGTCTCAGATGTCAG	506	611 to 1116
	AGACCTCAGTCGGCATCATACC	AAGATGGCCTCCTCCTCATCAC	1222	1588 to 2809
	TTGATGATTACCTCGCTGACCG	GCTTTGGCATTGGAACCAGTC	634	2740 to 3373
	TTGAGAACACCCTTGAACCGCAG	CCATCCTCGTTCATTGACATGCC	394	3826 to 4219
	GGTCTCGACCAACCTCGAATTG	GCGCCATCACTCATCCTCATTC	493	4583 to 5075
MNV-4	GGATGACTTCGGCATGGATAAC	TTGTTCTTGCCCTTCTTGCC	972	1664 to 2635
	CACTTTCACCAGTGCCGTTAG	CTTTGAGAGCTTCACAGAAGGG	1006	3162 to 4167

OneStep RT-PCR Kit (Qiagen, Valencia, CA) according to manufacturer's recommended protocol with thermocycling parameters consisting of reverse transcription at 50ºC for 30 min, activation of DNA polymerase at 95ºC for 15 minutes, 40 cycles of denaturation (94ºC, 1 min), annealing (55 ºC, 1 min), and extension (72ºC, 1 min), and a final extension at 72ºC for 10 min. Successful RT-PCR products were purified either by using Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA) or by separation on a 1.5% SeaPlaque GTG Agarose Gel (Cambrex, Rockland, ME) followed by purification using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). Purified RT-PCR products were sequenced in both directions by SeqWright DNA Technology Services (Houston, TX) and the sequence data obtained were used to design additional specific primers (Table 4.1) to fill in fragments corresponding to gaps in the nucleotide sequence. The termini of the MNV genomes were obtained using the 5' and 3' RACE Systems (Invitrogen, Carlsbad, Calif.) and "gene specific primers" (Table 4.2) as defined by the manufacturer's recommended protocol. Contig assembly was performed using Sequencher Software (Gene Codes Corp., Ann Arbor, Mich.) and edited manually to resolve any base call discrepancies by evaluating chromatograms.

Phylogenetic analysis. Full-length nucleotide sequence alignments and predicted amino acid sequence alignments of the polymerase and capsid proteins from human and animal noroviruses were generated using Clustal W within the Discover Studio Gene software package (Accelrys, San Diego, CA). Gaps in the nucleotide and amino acid sequence alignments were removed and phylogenetic trees were generated with DS Gene using the neighbor-joining method with the Tajima-Nei distance calculation for nucleotides or the Poisson Correction for amino acids. Bootstrap analysis was performed

Table 4.2. Primers used to amplify the 5' and 3' ends of novel murine noroviruses.

RACE[a] protocol	MNV strain	Primer[b]	Primer sequence (5' to 3')
5' RACE	MNV-2	GSP 1	AGTTTTGTCCTCCTTAGGCCG
		GSP 2	CGGCGGAAGATAGCCTTATCAG
		GSP 3	TAGCCACGGCTCAGACTTCTTC
	MNV-3	GSP 1	CTGCTCAAGGATAGACCCAATC
		GSP 2	TCCCTTGCATCCATAGCGTC
		GSP 3	TTATCTTCCTTGGGCCGCAG
	MNV-4	GSP 1	ACCACTGGCTCTCTTGCATC
		GSP 2	CCTTATCTTCCTTGGGTCGCAG
		GSP 3	ACGGAAGATGGCCCTATCAGAC
3' RACE	MNV-2	GSP 1	TTCAAAGTCCGACGCCATTC
	MNV-3	GSP 1	AGCCAACTCTTTCAAGCACGAC
	MNV-4	GSP 1	AACGCCATCAACATCAGGTC

[a]RACE = rapid amplification of cDNA ends

[b]GSP = gene specific primer

using 1000 replicates. Nucleotide and amino acid identity percentages were generated with DS Gene by generating a matrix using the uncorrected distance calculation method of the nucleotide and amino acid alignments after gaps were removed and calculating 1 minus the uncorrected distance. GenBank accession numbers for the human and animal noroviruses included in the alignments are Norwalk (M87661), Southampton (L07418), Desert Shield (U04469), Jena (AJ011099), Snow Mountain (AY134748), Hawaii (U07611), Lordsdale (X86557), Toronto (U02030), Fort Lauderdale (AF414426), murine norovirus 1 (AY228235.2), murine norovirus 2 (DQ223041), murine norovirus 3 (DQ223042), murine norovirus 4 (DQ223043), and the sapovirus Manchester (X86560).

RESULTS

RNA sequence analysis. To compare the genomes and molecular characteristics of the three novel murine noroviruses to that of MNV-1 and other human and animal noroviruses, the full-length nucleotide sequences of MNV-2, MNV-3 and MNV-4 were obtained by nucleotide sequencing RT-PCR and 5' and 3' RACE products. Percent nucleotide identities of the four MNV strains ranged from 87.4% to 91.5%, demonstrating that they are related but distinct strains of murine noroviruses (Table 4.3). The genome of MNV-1 contains 7382 nucleotides while the three novel MNV strains each contain 7383 nucleotides. The RNA genomes of all four MNV strains begin with a 5' GU and end with a poly-A tail, similar to other caliciviruses (63). The initial 32 nucleotides at the 5' end of all four MNV strains are identical and nucleotide position 33 of MNV-1 contains a C nucleotide instead of a U nucleotide as in the other MNV strains (Figure 4.1). When comparing the 5' ends of MNV-2, MNV-3, and MNV-4, the first

Table 4.3. Percent nucleotide identities of the full length genome after removal of gaps among human and animal noroviruses.

	Genogroup I		Genogroup II			Genogroup III	Genogroup V			
	Norwalk (%)	Southampton (%)	Snow Mountain (%)	Hawaii (%)	Lordsdale (%)	Jena (%)	MNV-1 (%)	MNV-2 (%)	MNV-3 (%)	MNV-4 (%)
Norwalk	*									
Southampton	73.7	*								
Snow Mountain	56.5	56.3	*							
Hawaii	56.5	56.8	79.6	*						
Lordsdale	55.6	55.8	77.4	80.3	*					
Jena	61.3	61.2	54.6	54.7	54.3	*				
MNV-1	52.4	52.3	53.7	53.4	53.2	52.5	*			
MNV-2	52.6	52.7	54.3	53.7	53.9	53.0	87.4	*		
MNV-3	52.4	52.3	54.4	54.0	54.0	53.3	87.7	91.5	*	
MNV-4	52.6	52.1	54.2	53.5	53.6	53.1	87.4	91.5	91.4	*

* = 100%

```
            1       10          20          30          40          50          60          70          80
MNV-1:      ---------- ---------- ---------- --C------- ---------- ---------- -------G-- -G---CG---
MNV-2:      ---------- ---------- ---------- --U------- ---------- ---------- -------A-- -A---CA---
MNV-3:      ---------- ---------- ---------- --U------- ---------- ---------- -------A-- -G---CA---
MNV-4:      ---------- ---------- ---------- --U------- ---------- ---------- -------A-- -G---UG---

Consensus:  GUGAAAUGAG GAUGGCAACG CCAUCUUCUG CGYCCUCUGU GCGCAACACA GAGAAACGCA AAAACAARAA GRCUUYRUCU

            5052                        5080
Subgenomic: ----*----- ----AGUGAU GGCG-AG-GC
```


Figure 4.1. Alignment and comparison of the first 80 nucleotides at the 5'-end of four MNV strains. The comparison of the 5' end of the genomic RNA consensus sequence with the 5' end of the predicted subgenomic RNA strand beginning at nucleotide position 5052 is also depicted. Dashed lines indicate identical nucleotides. Underlined nucleotides indicate in-frame AUG start codons. The asterisk (*) indicates the position of a gap that was inserted in the predicted subgenomic RNA sequence to enhance the alignment with the genomic RNA.

nucleotide variation occurs at nucleotide position 72 with an A nucleotide in the MNV-2 sequence (Figure 4.1). This highly conserved nucleotide sequence at the 5' end of all the MNV genomes is nearly identical in sequence to a region just upstream of the ORF2 start codon with an exact match of 13 nucleotides beginning at position 5052 when a single nucleotide gap is inserted (Figure 4.1). This conservation of the nucleotide sequences at the 5' end of the full-length genome to the region just upstream of ORF2 suggests that the expression of the structural proteins of ORF2 and ORF3 are initiated from subgenomic RNA (230, 231). All four of the MNV genomes are organized into three predicted ORFs with ORF1 spanning from nucleotide positions 6 to 5069, ORF2 from 5056 to 6681, and ORF3 from 6681 to 7307. The first AUG start codon of ORF1 begins at the sixth nucleotide position with a second AUG in the same reading frame beginning at nucleotide position 12 (underlined, Figure 4.1). A stop codon terminates ORF1 at nucleotide position 5069. There is a 14 nucleotide overlap between ORF1 and ORF2, creating a -2 frame shift of ORF2 compared to ORF1. A single nucleotide overlap is present at the termination codon of ORF2 and the start codon of ORF3, which places ORF3 back into the same reading frame as ORF1. At the end of ORF3, there is a 75 (MNV-1) or 76 (MNV-2, MNV-3 and MNV-4) nucleotide 3' untranslated region before the polyadenylated tail. The 3' untranslated region contains a high A-U nucleotide content ranging from 66.7% to 69.7%, as has been previously reported for some norovirus strains that infect humans (187).

When compared to available full-length nucleotide sequences for human norovirus strains of genogroup I and genogroup II, and for a bovine norovirus in

genogroup III, the MNV nucleotide sequences displayed percent nucleotide identities ranging from 52.1% to 54.4% (Table 4.3).

Predicted protein sequence analysis. To evaluate and characterize the protein sequences of the murine noroviruses, the three ORFs of the MNV genomes were translated and the predicted amino acid sequences analyzed. The polyprotein encoded by ORF1 contains 1687 amino acids, the capsid protein encoded by ORF2 contains 541 amino acids, and the small basic structural protein encoded by ORF3 contains 208 amino acids. The calculated molecular mass of the polyprotein, capsid protein, and small basic structural protein for all the MNV strains is 187 kDa, 58 kDa, and 22 kDa, respectively. The predicted polymerase protein and capsid protein amino acid sequences of the three novel MNV strains were compared to each other, MNV-1, and to noroviruses in genogroups I through IV. The polymerase amino acid sequences were slightly more conserved than the capsid amino acid sequences among the MNV strains with percent amino acid identities for the polymerase gene ranging from 98.1% to 99.3% (Table 4.4) and for the capsid gene ranging from 94.4% to 98.3% (Table 4.5). When the polymerase amino acid sequences of the MNV strains were compared to that of human and bovine noroviruses, percent amino acid identities ranged from 51.3% to 54.7% (Table 4.4). When the capsid amino acid sequences of the MNV strains were compared to those of human and bovine noroviruses, percent amino acid identities ranged from 37.8% to 41.3% (Table 4.5).

The capsid region was further analyzed by evaluating the percent amino acid sequence identities for the S and P domains. The S domain is involved in the formation of the icosahedral shell while the P domain forms the protruding arm extending from the

Table 4.4. Percent amino acid identities of the polymerase protein after removal of gaps among human and animal noroviruses.

	Genogroup I			Genogroup II				Genogroup III	Genogroup IV	Genogroup V			
	Norwalk (%)	Southampton (%)	Desert Shield (%)	Snow Mountain (%)	Hawaii (%)	Lordsdale (%)	Toronto (%)	Jena (%)	Fort Lauderdale (%)	MNV-1 (%)	MNV-2 (%)	MNV-3 (%)	MNV-4 (%)
Norwalk	*												
Southampton	92.9	*											
Desert Shield	82.5	83.6	*										
Snow Mountain	64.3	65.1	62.5	*									
Hawaii	64.3	65.1	63.9	95.5	*								
Lordsdale	63.6	64.3	63.2	96.7	97.4	*							
Toronto	62.8	63.9	61.3	92.2	91.1	91.1	*						
Jena	73.6	71.8	73.6	65.8	65.8	65.4	64.3	*					
Fort Lauderdale	63.2	61.7	61.7	71.8	72.1	72.5	72.5	65.8	*				
MNV-1	53.2	54.3	53.5	53.5	52.8	52.8	53.9	53.2	51.3	*			
MNV-2	53.9	54.3	53.9	53.9	53.2	53.2	54.3	53.9	51.7	98.1	*		
MNV-3	53.9	54.3	53.9	53.9	53.2	53.2	54.3	53.9	51.7	98.9	99.3	*	
MNV-4	53.2	53.5	53.5	54.3	53.5	53.5	54.7	53.9	52.0	98.1	98.5	98.5	*

* = 100%

Table 4.5. Percent amino acid identities of the capsid protein after removal of gaps among human and animal noroviruses.

	Genogroup I			Genogroup II				Genogroup III	Genogroup IV	Genogroup V			
	Norwalk (%)	Southampton (%)	Desert Shield (%)	Snow Mountain (%)	Hawaii (%)	Lordsdale (%)	Toronto (%)	Jena (%)	Fort Lauderdale (%)	MNV-1 (%)	MNV-2 (%)	MNV-3 (%)	MNV-4 (%)
Norwalk	*												
Southampton	72.0	*											
Desert Shield	68.9	67.6	*										
Snow Mountain	45.7	42.2	45.0	*									
Hawaii	45.5	43.6	44.6	77.2	*								
Lordsdale	43.1	42.8	43.0	65.3	66.3	*							
Toronto	45.9	41.4	44.0	71.8	73.5	69.4	*						
Jena	50.0	48.2	47.9	45.0	45.2	46.0	45.7	*					
Fort Lauderdale	44.1	43.6	43.0	53.0	53.7	52.3	52.7	42.9	*				
MNV-1	40.6	40.0	38.8	41.1	39.2	40.5	38.5	41.3	40.9	*			
MNV-2	40.4	39.5	38.2	40.9	39.8	40.2	38.7	40.9	41.0	94.4	*		
MNV-3	40.4	39.5	37.8	40.5	40.0	39.8	38.5	40.5	40.9	94.4	98.3	*	
MNV-4	40.6	39.5	37.8	40.7	40.2	40.0	38.7	40.7	41.1	94.4	98.3	98.1	*

* = 100%

shell (188). Percent amino acid identities for the S domain among all four MNV strains ranged from 99.1% to 100%, while the P domain was slightly less conserved, ranging from 90.8% to 97.5% (Table 4.6). When the S and P domains of the MNV strains were compared to the amino acid sequences of these domains from both human and bovine noroviruses, the percent amino acid identities for the S domain ranged from 53.6% to 58.2% while the P domain was less conserved, ranging from 25.4% to 32.8% (Table 4.6).

Phylogenetic analysis. To examine the phylogenetic clustering of the novel MNV strains with other noroviruses including the previously reported MNV-1, phylogenetic trees were generated using available full-length nucleotide sequences, the predicted polymerase amino acid sequence, or the predicted capsid amino acid sequence using murine, bovine, and human norovirus sequences. Phylogenetic trees that were produced in all cases displayed similar groupings with respects to genogroup clustering with the murine noroviruses grouping together and separate from the human and bovine noroviruses. A representative phylogenetic tree generated using the predicted amino acid sequence of the capsid protein is shown in Figure 4.2.

DISCUSSION

Although many strains of noroviruses have been described that infect humans, only one strain of a mouse specific norovirus, MNV-1, was reported in 2003 (106). Subsequent to the discovery of MNV-1, our laboratory isolated three novel murine norovirus strains, MNV-2, MNV-3 and MNV-4, from the mesenteric lymph nodes of laboratory mice (85). We reported that these three novel MNV strains differed greatly in pathogenicity to MNV-1 by causing persistent infections and prolonged fecal shedding in

Table 4.6. Percent amino acid identities of the S and P domains of the capsid region in murine, bovine and human noroviruses.

	S Domain				P Domain			
	MNV-1 (%)	MNV-2 (%)	MNV-3 (%)	MNV-4 (%)	MNV-1 (%)	MNV-2 (%)	MNV-3 (%)	MNV-4 (%)
MNV-2	99.6				90.8			
MNV-3	99.6	100.0			90.8	97.1		
MNV-4	99.1	99.6	99.6		91.1	97.5	97.2	
Norwalk	55.2	55.2	55.2	55.2	29.4	29.1	29.1	29.4
Southampton	57.7	57.7	57.7	58.2	26.7	25.7	25.7	25.4
Desert Shield	54.1	53.6	53.6	53.6	26.8	26.1	25.5	25.5
Snow Mountain	57.0	57.0	57.0	57.0	29.8	29.6	28.8	29.1
Hawaii	56.6	56.6	56.6	56.6	27.2	28.2	28.5	28.8
Lordsdale	53.9	53.9	53.9	53.9	31.5	31.3	30.5	30.5
Toronto	53.9	53.9	53.9	53.9	27.9	28.3	27.9	28.2
Jena	53.7	53.7	53.7	53.7	32.8	32.1	31.4	31.7
Fort Lauderdale	56.1	56.1	56.1	56.1	30.5	30.9	30.8	30.8

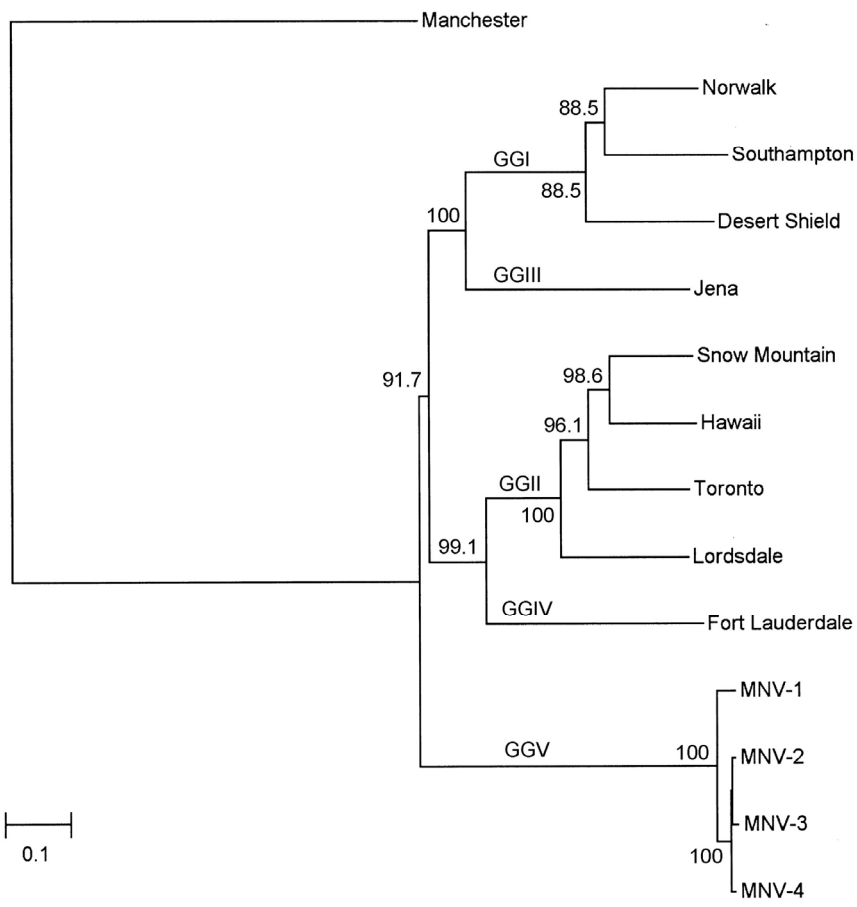

Figure 4.2. Phylogenetic tree generated by using the neighbor-joining method and the predicted capsid amino acid sequences of human and animal noroviruses. Trees produced using available full-length nucleotide sequences or the predicted polymerase amino acid sequences of human and animal noroviruses displayed similar genogroup clustering. Bootstrap values are given for each node based on 1000 replicates. The Manchester strain of the genus *Sapovirus* was used as the outgroup. The scale represents the number of changes per site. GGI, genogroup I; GGII, genogroup II; GGIII, genogroup III; GGIV, genogroup IV; GGV, genogroup V.

infected laboratory mice, whereas MNV-1 induced only a transient infection and short duration of fecal shedding (84, 85, 106). Herein, we describe the molecular characterization of these novel MNV isolates to confirm that these isolates are related to MNV-1, but that they are also genetically distinct from MNV-1 and from one another to warrant their classification as unique MNV strains.

Comparisons of the full-length nucleotide sequence identities of all four MNV strains with each other revealed a high degree of sequence homology with each other but low sequence homology with human and bovine noroviruses. Phylogenetic analysis likewise demonstrated clustering of MNV-2, MNV-3 and MNV-4 with the previously described MNV-1 as a single genogroup, genogroup V, separate from the other human and animal norovirus genogroups. Karst *et al.* previously described similar phylogenetic clustering when comparing MNV-1 to other noroviruses (106). This phylogenetic clustering of the MNV strains together as a single genogroup was replicated when the analysis was conducted using either full-length nucleotide sequences, predicted capsid amino acid sequences, or predicted polymerase amino acid sequences. These data indicate that MNV-2, MNV-3 and MNV-4 are related to MNV-1 even though the pathogenicity of MNV-2, MNV-3 and MNV-4 are markedly different from that of MNV-1.

Additional analysis of the MNV genomes revealed many similarities to the genomes of other noroviruses, further supporting their classification within this genus. These similarities include the size of the genome, a 5' end terminal GU, a short 5' untranslated region, multiple in-frame AUG start codons at the 5' end, three open reading frames encoding a polyprotein, capsid protein and small basic structural protein, a 3'

untranslated region, and a poly-adenylated tail (63). There is also conservation of the 5' end sequence motif of the genomic RNA with a site just upstream of the start codon of ORF2. This conserved motif upstream of ORF2 corresponds to a potential transcription initiation site for a subgenomic RNA strand, as has been reported for other caliciviruses (7, 63, 153). The production of subgenomic RNA is important during calicivirus infections since the subgenomic RNA, rather than the genomic RNA, is used as the template for production of the capsid protein (7, 63, 203).

Amino acid sequence analysis of the four MNV strains demonstrated that the polymerase protein sequences were more highly conserved than the capsid protein sequences which demonstrated greater variability. A similar trend was found when murine, human, and bovine norovirus amino acid sequences were aligned and evaluated. These findings are not unexpected as mutations in the polymerase portion of the genome may be more likely to result in a non-functional protein that is deleterious for the virus, while mutations in the structural capsid protein may be less likely to be deleterious and may even serve as a method of immune evasion. Analysis of the capsid protein amino acid sequences of the S and P domains demonstrated that the S domain is more highly conserved than the P domain, as has been shown with other noroviruses (63, 73, 221). These results are not unexpected since the S domain forms the inner icosahedral shell while the P domain serves as the major antigenic site forming the protruding portions of the capsid protein that are more exposed than the S domain to the selective pressures of the host immune system (63, 188).

In summary, three novel, genetically distinct, murine norovirus strains were isolated and characterized, and these strains exhibited many of the molecular and genetic

features consistent with caliciviruses, supporting their classification as murine noroviruses. In addition, the three novel murine norovirus strains demonstrated a markedly different pathogenicity from the previously reported MNV-1 but were confirmed by phylogenetic analysis to cluster with MNV-1 and separate from other human and animal noroviruses.

CHAPTER V

Cytokine and Chemokine Response in Mice Infected with Murine Norovirus

INTRODUCTION

It is estimated that in the United States there are more than 23 million cases of human norovirus infections each year (146). These infections cause clinical symptoms such as nausea, vomiting, diarrhea, fever, and abdominal cramps that typically last for 24 to 48 hours (36, 65). Many of these infections occur in nursing homes, hospitals, cruise ships, restaurants, schools, or other areas where close contact aids in the spread of the virus (45, 46, 93, 135, 136). In addition, noroviruses account for as much as 67% of food-related illnesses among all the know foodborne bacterial, parasitic and viral pathogens (146, 229), and have been responsible for greater than 95% of outbreaks of nonbacterial gastroenteritis (45, 46).

Although noroviruses are a significant cause of gastroenteritis worldwide, the immune response to norovirus infection is poorly understood since, until recently, there was no small animal model to study the disease and noroviruses could not be grown in cell culture (42, 230, 231). In 2003, a norovirus infecting laboratory mice (murine norovirus, MNV) was discovered, found to propagate in vitro, and used to characterize

parts of the immune response to norovirus infection (85, 106, 157, 230). In these studies, it was found that two components of the innate immune system, STAT1 and interferon (IFN)-αβγ receptors, were essential to protect mice against lethal MNV infection (106). MNV infection also stimulated the production of the innate cytokine IFN-α in cultured mouse bone-marrow-derived macrophages and dendritic cells (230), as well as IFN-β in the serum and jejunum of infected mice (157). Mice infected with MNV also exhibited a robust IgG antibody response (84, 85) indicating activation of the adaptive immune response during infection. However, since mice become persistently infected with most strains of MNV (85), the immune response is ultimately ineffective in clearing MNV infection.

In humans, limited information is available on the immune response to norovirus infection since there still is no permissive cell line to propagate human noroviruses (42). Therefore, data have been mostly derived from human volunteer challenge studies with norovirus infected fecal filtrates or virus-like particles (57, 127, 209). In these studies, human volunteers inoculated with noroviruses or virus-like particles have increases of T helper 1 (Th1) cytokines such as gamma interferon (IFN-γ) and interleukin (IL)-2 in the absence of the Th2 cytokines IL-4, IL-5 and IL-10 (120, 127, 209). Short-term immunity develops in inoculated volunteers (36, 100, 141, 182) but interestingly, long term immunity is not induced since some people became ill when rechallenged 27 to 42 months later (141, 182). Paradoxically, volunteers with preexisting antibody titers were not protected against illness but rather were more likely to develop clinical signs of disease than those with low antibody titers who were resistant to infection (57, 100, 141,

173, 182). These resistant volunteers failed to develop a serologic response even after multiple challenges with noroviruses (100, 182).

In light of the immunologic changes caused by norovirus infections described thus far, there is the potential that using MNV infected laboratory mice in research studies unrelated to norovirus pathogenesis may lead to alterations in research data due to these unwanted immunologic changes. In support of this, it has been previously shown that laboratory mice subclinically infected with pathogens such as mouse parvovirus, mouse hepatitis virus, lactate dehydrogenase-elevating virus, mouse adenovirus, *Clostridium piliforme*, or *Helicobacter* sp. have alterations in their immunologic physiology as a result of these infections (9, 31, 145, 159, 164, 216). These immunologic perturbations may be devastating if they alter the outcome of research data and lead to false observations or conclusions when using infected mice (9, 145, 164). Additionally, antibodies to MNV have been documented in 22.1% of 12,639 serum samples from laboratory mice submitted to our diagnostic laboratory (84) indicating that a large proportion of laboratory mice harbor MNV as a potential confounding factor in biomedical research studies. To date, no clinical signs of disease have been attributed to MNV infection in immunocompetent and most immunodeficient strains of mice (85, 106), but subclinical alterations of the immune system may still be occurring in these mice.

Therefore, the goals of this study were to characterize the immunologic perturbations that occur in laboratory mice infected with MNV when compared to uninfected mice. We used focused microarrays capable of simultaneously screening 113 key genes of the inflammatory response to gain a generalized overview of the changes in

gene expression in MNV infected mice. We then used quantitative real-time RT-PCR to confirm the immunomodulatory effects identified by the microarray. In addition, ELISAs were used to detect soluble protein expression of type I interferons in the sera of infected mice. The results obtained from this study would provide further information on the pathogenesis of MNV infection in laboratory mice, assisting biomedical researchers in determining the impact of using MNV infected mice in their research studies. In addition, these studies would provide additional comparative data to help elucidate the immune response to norovirus infections in humans.

MATERIALS AND METHODS

Animal inoculations and sample collection. Groups of 4- to 5-week-old female BALB/cAnNCr and C57BL/6NCr mice (National Cancer Institute, Frederick, MD) were received, acclimated for 1 week, and inoculated at 5- to 6-weeks-old. Mice were verified by vendor surveillance to be free of ectoparasites, endoparasites, *Mycoplasma pulmonis*, *Helicobacter* sp., known enteric and respiratory bacterial pathogens, and antibodies to mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler's murine encephalomyelitis virus, ectromelia virus, polyoma virus, K virus, lymphocytic choriomeningitis virus, Hantaan virus, mouse adenovirus, minute virus of mice, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, *Encephalitozoon cuniculi*, *Clostridium piliforme*, Cilia Associated Respiratory Bacillus, and murine norovirus. Feces from a subset of mice were also collected prior to inoculation and verified to be free of MNV by RT-PCR as previously described (85). Mice were group-housed in static filter top cages according to infection status and were

provided autoclaved, acidified water and standard irradiated rodent chow *ad libitum*. All mouse manipulations were performed in a class II biological safety cabinet and studies were approved by the University of Missouri's Animal Care and Use Committee.

Propagation of MNV-4 in RAW 264.7 cells was performed as previously described (84, 85). At 5- to 6-weeks old, experimental mice were inoculated with 5×10^6 to 1×10^7 PFU of clarified MNV-4-infected RAW cell lysates by oral gavage. Control mice were sham-inoculated with similar volumes of clarified uninfected RAW cell lysates. For BALB/c mice, mice were euthanized with an overdose of inhaled carbon dioxide and samples collected at day 1 post-infection (PI), day 3 PI, and week 4 PI. For C57BL/6 mice, mice were euthanized and samples collected at week 4 PI. Blood was collected by cardiocentesis, allowed to clot at room temperature for 15 to 30 minutes, and the serum separated by centrifugation at 2000 × g at 4°C, divided into 50 µl aliquots on ice, and stored at -80°C until use. Mesenteric lymph nodes (MLN) and jejunum samples approximately 0.5 cm long and 6 to 8 cm from the ileocecal junction were collected, placed in 0.5 ml of RNAlater (Ambion, Austin, TX) and kept at 4°C overnight. After the overnight incubation, the RNAlater was removed and samples stored at -80°C until use.

Microarray analysis. MLN and jejunum samples were homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) using 5-mm stainless steel balls and a TissueLyser (Qiagen, Valencia, CA) at 30 Hz for 2 minutes twice. RNA was then extracted according to manufacturer's recommended protocol and A_{260} and A_{280} were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). To remove any genomic DNA contamination, 20 µg of RNA was DNase treated in 100 µl reaction volumes using TURBO DNA-free (Ambion, Austin, TX) according to

manufacturer's recommended protocol. Samples were then purified using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA) using the manufacturer's recommended protocol for purifying RNA from liquid samples. RNA concentration and purity were determined using the NanoDrop spectrophotometer. RNA integrity was evaluated by electrophoresis of approximately 400 ng of RNA in a 1% SeaKem GTG agarose gel (Cambrex, Rockland, ME) at 80V for 30 minutes and examining the 28S and 18S ribosomal RNA bands under ultraviolet light for smearing as an indicator of RNA degradation.

Microarrays were performed using the Mouse Inflammatory Cytokines and Receptors Oligo GEArray DNA Microarray (SuperArray Bioscience Corp., Frederick, MD) according to manufacturer's recommended protocol. Two micrograms of starting RNA was used for the initial cDNA synthesis and then the cRNA synthesis reaction was incubated overnight. Each nylon membrane microarray was hybridized to 3 μg of biotin-labeled cRNA target derived from the tissue of a single mouse, i.e. samples were not pooled. Images of each microarray were acquired using a Kodak Image Station 4000R cooled CCD camera (Eastman Kodak, Rochester, NY) with the settings: zoom 85, F stop open, focus 0, no binning, and 20 minute exposure time. Images were uploaded and evaluated with the GEArray Expression Analysis Suite 2.0 (SuperArray Bioscience Corp., Frederick, MD). Volcano plots were generated with the settings: average density, clover off, empty spot background correction, 1.50 AP threshold, and normalization to heat shock protein 1 beta. On the microarray, a single gene was represented by a spot group consisting of 4 individual spots. Therefore, genes were considered to have a change in expression level if a spot group from MNV-4 infected experimental mice (n=3)

had a greater than 2-fold increase or decrease in density when compared to the same spot group from sham-inoculated control mice (n=3) and had a *P* value less than 0.01.

Quantitative real-time RT-PCR analysis. MLN and jejunum samples were homogenized as described above and the RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommended protocol. The SuperScript First-Strand Synthesis System for RT-PCR kit with the Oligo(dT) primer (Invitrogen, Carlsbad, CA) was used to create first-strand cDNA from 2 µg of RNA and the resulting cDNA diluted to a final concentration of 20 ng/µl. Real-time PCR was performed in duplicate using the LightCylcler 1.5 with software version 4.0 (Roche Diagnostics, Basel, Switzerland), and the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) in 20 µl capillaries. For IFN-α and IFN-β, 180 ng of starting template cDNA was used for each real-time PCR assay, while 80 ng of cDNA was used for all other assays. To account for the variability in sample preparation, RNA isolation, and first-strand cDNA synthesis, the expression level of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was measured in each sample and used to normalize the expression level of each gene. Primer pairs used to amplify genes involved in the inflammatory response and HPRT were obtained from previous reports in the literature, developed using the Discover Studio Gene software package version 1.5 (Accelrys, San Diego, CA), or ordered from Qiagen Gene Globe QuantiTect Primer Assays (Qiagen, Valencia, CA) (Table 5.1). Thermocycling parameters, $MgCl_2$ concentrations (2.5 mM or 3.0 mM), and ramp rates varied and were based on previous reports (159, 160) or Qiagen's recommended protocols. All primer pairs flanked introns, spanned intron/exon boundaries, or the RNA was DNase treated and spin column purified

Table 5.1. Primers used for quantitative real-time RT-PCR amplification.

Gene Name	Primer sequence (5' to 3')		Annealing Temp. (°C)	Amplicon size (bp)	Reference
	Forward	Reverse			
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA	60	177	(159, 160, 171)
IFN-α4	GAAGGCTCAAGCCATCCTTGTG	AGCTGCTGATGGAGGTCATTGC	59	132	NM_010504
IFN-β	CCAGCTCCAAGAAAGGACGAAC	CTGCATCTTCTCCGTCATCTCC	59	124	NM_010510
IFN-γ	GCCATCAGCAACAACATAAGC	TGGGACAATCTCTTCCCCAC	58	238	(160)
TNF-α	AAGTTCCCAAATGGCCTCCCTC	TCCACTTGGTGGTTTGCTACG	60	119	C. Patten unpublished
CXCL9 (MIG)	ACTCGGCAAATGTGAAGAAG	CCCATTAAGATTCAGGGTGC	58	194	(160)
CXCL10 (IP-10)	CCGGAATCTAAGACCATCAAG	GTCACCTTTCAGAAGACCAAG	60	194	(160)
CCL5 (RANTES)	GAGTATTTCTACACCAGCAGC	GGACTAGAGCAAGCAATGAC	60	192	M. Myles unpublished
CCL19 (MIP-3β)	GTGCCTGCTGTTGTGTTCAC	GGTGCTGTTGCCTTTGTTCTTG	57	126	NM_011888
CCL21a	TGCCTTAAGTACAGCCAGAAG	CCGGGGTAAGAACAGGATTG	58	108	NM_011335
IL-1β	AGCCCATCCTCTGTGACTCATG	GCTGATGTACCAGTTGGGGAAC	57	422	(122, 160)
IL-10	GCCAAGCCTTATCGGAAATG	ATCACTCTTCACCTGCTCC	58	197	M. Myles unpublished
IL-10Rβ	Not available	Not available	55	109	Qiagen
CCR1	Not available	Not available	55	139	Qiagen

prior to cDNA synthesis (e.g. IFN-α and IFN-β) as described above to ensure that only mRNA and not genomic DNA was amplified. Melting curves were performed following amplification to verify PCR product identity.

To quantify the expression copy number of each inflammatory gene or HPRT in a sample, plasmid standards for each primer pair were generated. To make each plasmid, PCR amplicons were created using either the OneStep RT-PCR kit (Qiagen, Valencia, CA) or the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA), cloned using the TOPO TA Cloning Kit for Sequencing with One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA), and purified with the FastPlasmid Mini kit (Eppendorf, Hamburg, Germany). Quantification of plasmid copy number was calculated based on the DNA concentration using the NanoDrop and the estimated molecular weight of 660 daltons per base pair. Plasmids were linearized using the NotI restriction endonuclease and stored in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). Standard curves with know concentrations of each plasmid (10^1 to 10^6 copies) were generated and used to determine the copy number in each sample.

Statistical analysis was performed comparing normalized gene expression levels between experimental (n=10) and control (n=10) mice at each time point by using the Student's t-test (SigmaStat 3.5; Systat Software, Inc., Point Richmond, CA). A *P* value of less than 0.05 with a greater than 2-fold change in expression was considered biologically significant.

Type I interferon protein analysis in serum by ELISA. The Mouse Interferon Alpha and Beta ELISA kits (PBL Biomedical Laboratories, Piscataway, NJ) were used according to manufacturer's recommended protocol to quantify IFN-α and IFN-β levels

in serum from experimental (n=5) and control (n=5) BALB/c mice at day 1 and day 3 PI. Serum samples were assayed in duplicate at a 1:20 dilution. Standard curves were generated using Microsoft Excel 2003 (Microsoft, Redmond, WA).

RESULTS

Mice and infection status. To confirm MNV infection in BALB/c and C57BL/6 mice maintained for 4 weeks PI, serologic and fecal RT-PCR assays were performed as previously described (84, 85). All MNV inoculated experimental mice (BALB/c, n=13; C57BL/6, n=13) produced a robust antibody response to MNV when measure by the multiplex fluorescent immunoassay at 4 weeks PI. In addition, fecal samples were collected weekly and tested by RT-PCR to confirm active shedding of MNV. All fecal samples at all time points were positive for MNV by RT-PCR indicating that both BALB/c and C57BL/6 mice persistently shed MNV in the feces after experimental inoculation for the duration of the 4 week experiment. All control animals were negative for MNV by serology at 4 weeks PI and by fecal RT-PCR at all time points.

In BALB/c mice that were maintained for 1 day and 3 days after inoculation with MNV, RT-PCR on the MLN was performed to assess viral infection and trafficking to a major lymph node draining the gastrointestinal tract. Serologic assays were not performed since there was not sufficient time to allow for a detectable antibody response. In addition, RT-PCR on fecal or gastrointestinal tissue samples was not performed since a positive result may be due to pass through of the inoculum and not necessarily due to active viral infection. At day 1 PI, MNV was not detected by RT-PCR in any of the

MLN samples of MNV inoculated mice (n=16). At day 3 PI, 7 of 16 (43.8%) MLN samples were positive for MNV by RT-PCR. All control samples were negative.

Microarray analysis of inflammatory genes. To identify the immune response that occurs in laboratory mice after infection with MNV, focused microarrays were used to distinguish altered expression of 113 key genes involved in the inflammatory response. The microarrays consisted of nylon membranes spotted with gene-specific, 60-mer oligonucleotides to detect the mRNA expression of cytokines, chemokines and their receptors. Only genes with greater than a 2-fold change and a *P* value of less than 0.01 when compared to sham-inoculated control mice were considered altered by MNV infection. The MLN of BALB/c mice were evaluated by microarray analysis after acute infection (day 1 and day 3 PI) and after chronic infection (week 4 PI) with MNV. At day 1, day 3, and week 4 PI, there was increased expression of 2, 6, and 3 genes, respectively, in the MLN of MNV infected BALB/c mice when compared to uninfected control mice (Table 5.2). No genes were found to have decreased expression in the MLN of MNV infected BALB/c mice at any time point.

In C57BL/6 mice, the MLN and jejunum were evaluated by microarray analysis only after a chronic infection (week 4 PI) with MNV. In the jejunum, only 1 gene, CCL9 (macrophage inflammatory protein-1-gamma, MIP-1γ), was found to have decreased expression in MNV infected mice when compared to uninfected control mice (Table 5.3). No other genes were found to have changes in expression in the MLN or jejunum of MNV infected C57BL/6 mice at 4 weeks PI by microarray analysis.

Validation of microarray findings by quantitative real-time RT-PCR. To confirm the changes in gene expression identified by the microarray, a subset of these

Table 5.2. Inflammatory gene expression changes measured by microarray and quantitative real-time RT-PCR analysis in the mesenteric lymph nodes of MNV-4 infected BALB/c mice at day 1, day 3 and week 4 post-inoculation.

Time post-inoculation	Up-regulated genes		Down-regulated genes	
	Microarray	Real-time RT-PCR	Microarray	Real-time RT-PCR
Day 1	CCL19 (MIP-3β)	Not significant, $P > 0.05$	None	Not done
	CCL21a	Not significant, $P > 0.05$		
Day 3	CXCL10 (IP-10)	Significant, $P < 0.05$[a]	None	Not done
	CCL19 (MIP-3β)	Not significant, $P > 0.05$		
	CCL21a	Not significant, $P > 0.05$		
	CCL11 (eotaxin)	Not done		
	IL-1R1	Not done		
	Lymphotoxin a	Not done		
Week 4	CCL21a	Not significant, $P > 0.05$	None	Not done
	IL-10Rβ	Not significant, $P > 0.05$		
	C3	Not done		

[a]1.48-fold increase

Table 5.3. Inflammatory gene expression changes measured by microarray and quantitative real-time RT-PCR analysis in the mesenteric lymph nodes and jejunum of MNV-4 infected C57BL/6 mice at week 4 post-inoculation.

Tissue	Up-regulated genes		Down-regulated genes	
	Microarray	Real-time RT-PCR	Microarray	Real-time RT-PCR
MLN[a]	None	Not done	None	Not done
Jejunum	None	Not done	CCL9 (MIP-1γ)	Not done

[a]MLN, mesenteric lymph node

genes were evaluated by quantitative real-time RT-PCR. To strengthen biological significance, assays were performed on tissue samples from additional mice inoculated with MNV and not on the same samples used in the microarray analysis. Gene expression levels were normalized to HPRT for more accurate comparisons between samples. Genes that were tested included CCL19 (macrophage inflammatory protein-3-beta, MIP-3β), CCL21a, CXCL10 (interferon inducible protein-10, IP-10), and interleukin 10 receptor beta (IL-10Rβ). In the MLN of BALB/c mice, none of these genes evaluated, with the exception of CXCL10, had a statistically significant change in gene expression (defined as a $P < 0.05$) when measure by quantitative real-time RT-PCR that could confirm microarray findings (Table 5.2 and Figure 5.1, A through D). Although CXCL10 at day 3 PI had a $P < 0.05$, the fold increase in expression was only 1.48-fold (Figure 5.1A), which was less than the defined threshold of at least a 2-fold change in expression to be considered biologically significant.

In light of the difficulties confirming microarray findings using quantitative real-time RT-PCR, additional genes considered unaltered by the microarray in MNV infected mice were evaluated using quantitative real-time RT-PCR on tissue samples from either BALB/c or C57BL/6 mice. Although these genes were not considered altered by the microarray, it was hypothesized that changes in gene expression may still be uncovered by quantitative real-time RT-PCR since this assay has been reported to have a greater sensitivity than microarrays in gene expression analyses (180, 224). Genes that were tested included those that have been previously reported to be involved in the inflammatory response during viral infections (IFN-γ, IL-1β, TNF-α, IL-10, CXCL9 and CCL5) (2, 18, 53, 132, 152), those that would be considered altered by the microarray

Figure 5.1

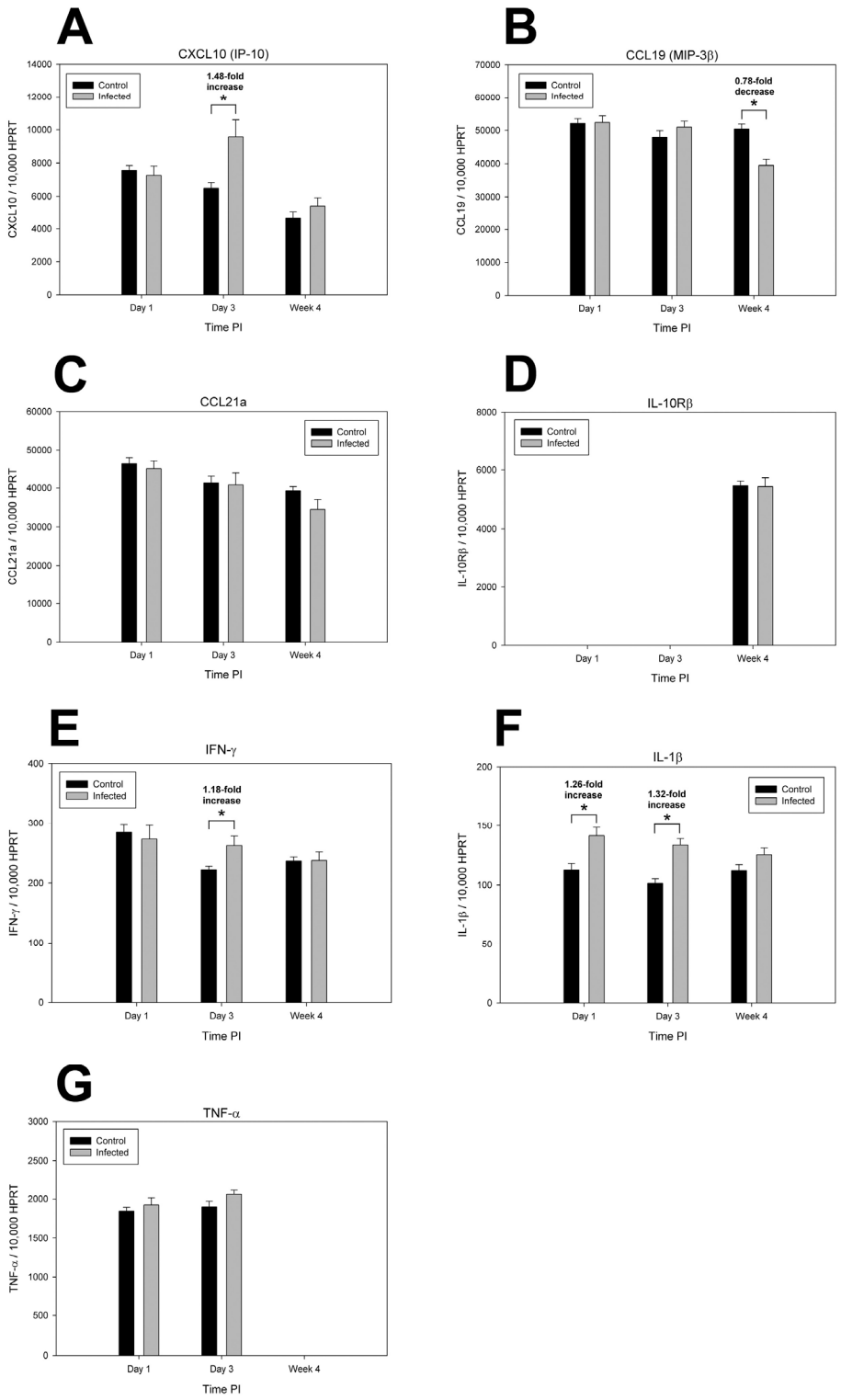
A
CXCL10 (IP-10)
CXCL10 / 10,000 HPRT
Control
Infected
1.48-fold increase
*
Day 1
Day 3
Week 4
Time PI
B
CCL19 (MIP-3β)
CCL19 / 10,000 HPRT
0.78-fold decrease
C
CCL21a
CCL21a / 10,000 HPRT
D
IL-10Rβ
IL-10Rβ / 10,000 HPRT
E
IFN-γ
IFN-γ / 10,000 HPRT
1.18-fold increase
F
IL-1β
IL-1β / 10,000 HPRT
1.26-fold increase
1.32-fold increase
G
TNF-α
TNF-α / 10,000 HPRT

Figure 5.1. Gene expression levels of CXCL10 (A), CCL19 (B), CCL21a (C), IL-10Rβ (D), IFN-γ (E), IL-1β (F), and TNF-α (G) in the mesenteric lymph nodes of BALB/c mice infected with MNV-4 at day 1, day 3 and week 4 post-inoculation. Bar heights represent the mean number of mRNA molecules relative to the number of HPRT mRNA molecules, and error bars represent the standard error of the mean. A $P < 0.05$ and a 2-fold or greater change in gene expression (infected/control; 2.0 for ↑ expression, 0.5 for ↓ expression) was considered biologically significant. *, $P < 0.05$.

when using a less stringent microarray *P* value of 0.05 (CCR1), or those that have been previously shown to have increased protein expression in MNV infected cell cultures (IFN-α) or mice (IFN-β) (157, 230). Of the additional inflammatory cytokine and chemokine genes tested by quantitative real-time RT-PCR, only the type I interferons, IFN-α4 and IFN-β, demonstrated a significant increase ($P < 0.05$) with a greater than 2-fold change in gene expression in the MLN of MNV infected BALB/c mice at day 3 and week 4 PI when compared to controls (Figure 5.2). Of note, the microarray included a spot group to detect IFN-α2, but was not considered significantly alerted in MNV infected mice; the microarray did not include a spot group to detect IFN-β. All other genes evaluated in BALB/c mice by quantitative real-time RT-PCR did not have biologically significant changes in gene expression (both a $P < 0.05$ and a greater than 2-fold change) (Figure 5.1, E through G). Likewise, in the tissues of MNV infected C57BL/6 mice at week 4 PI, no genes evaluated had both a $P < 0.05$ and a greater than 2-fold change in gene expression to be considered biologically significant (Table 5.4).

Serum protein ELISA for type I interferons. Since quantitative real-time RT-PCR indicated that IFN-α4 and IFN-β had increased gene expression in MNV infected mice and day 3 and week 4 PI, ELISAs were performed on sera from MNV infected and uninfected mice to corroborate these findings. Additionally, a previous study reported increases in serum IFN-β protein expression peaking at 24 hours in MNV-1 infected strain 129 mice (157). However, in this study, no IFN-α4 or IFN-β protein could be detected by ELISA in the sera of MNV-4 infected BALB/c mice at day 1 and day 3 PI. In light of these negative results, ELISAs were not performed on sera from mice at week 4 PI.

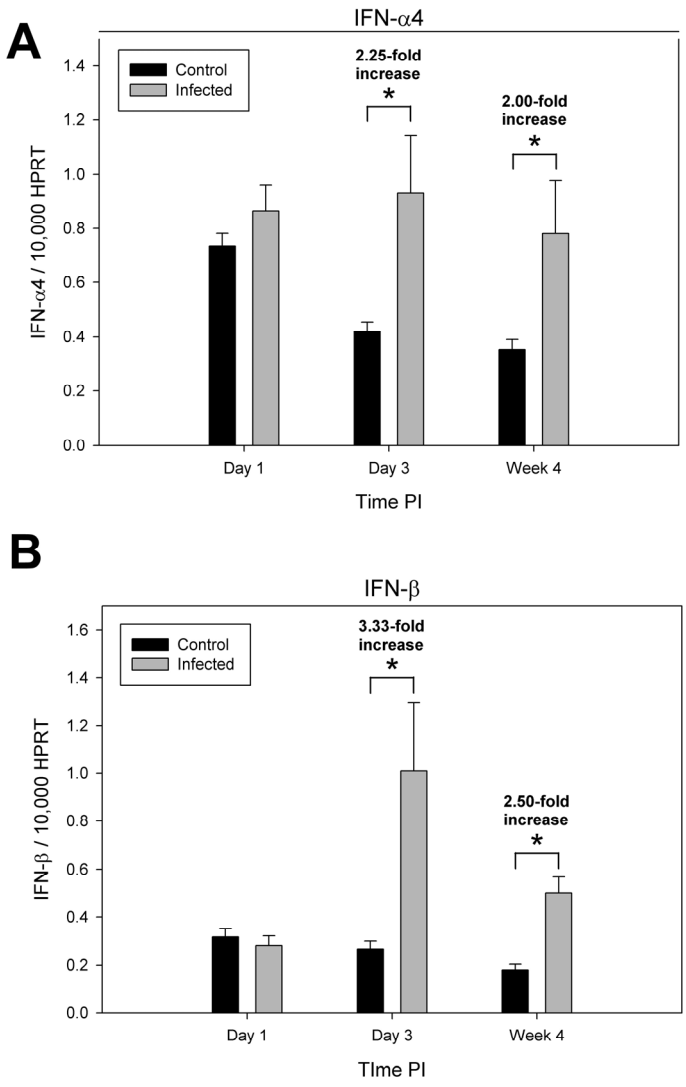

Figure 5.2. Gene expression levels of IFN-α4 (A) and IFN-β (B) in the mesenteric lymph nodes of BALB/c mice infected with MNV-4 at day 1, day 3 and week 4 post-inoculation. Bar heights represent the mean number of mRNA molecules relative to the number of HPRT mRNA molecules, and error bars represent the standard error of the mean. A $P < 0.05$ and a 2-fold or greater change in gene expression (infected/control; 2.0 for ↑ expression, 0.5 for ↓ expression) was considered biologically significant.

*, $P < 0.05$.

Table 5.4. Additional quantitative real-time RT-PCR assays performed on tissue samples from C57BL/6 mice at week 4 post-inoculation.

Mesenteric lymph node			Jejunum		
Gene Name	$P < 0.05$[a]	Fold change[a]	Gene Name	$P < 0.05$[a]	Fold change[a]
CCR1	Yes	0.77 ↓	CCR1	No	
IFN-γ	Yes	1.23 ↑	IFN-γ	No	
IL-10	No		IL-1β	No	
CCL5 (RANTES)	No		CXCL9 (MIG)	No	
			CXCL10 (IP-10)	No	
			TNF-α	No	

[a]A $P < 0.05$ and a 2-fold or greater change in gene expression (infected/control; 2.0 for ↑ expression, 0.5 for ↓ expression) was considered biologically significant.

DISCUSSION

The immunologic response to norovirus infection remains poorly understood. In humans norovirus infections, this lack of information has been due to the inability to propagate virus in a permissive cell line to generate reagents for experimental study (42). Additionally, while noroviruses have been discovered in swine and cattle (73, 221), these large animals are less amenable to comparative studies than a smaller laboratory animal species such as mice. With the recent recognition of a norovirus in laboratory mice and the discovery of an immortalized mouse macrophage cell line to propagated virus (106, 230), researchers now have a small animal model to study the pathogenesis of norovirus infection. Of equal importance however, is determining the effects that MNV may have in research studies unrelated to norovirus biology but that inadvertently use MNV infected mice. Since infection with MNV in mice used in biomedical research is so widespread (84), a significant proportion of research experiments may be confounded by these infections.

To address these concerns, the immunologic response to MNV infection in mice was evaluated using microarrays and validated by quantitative real-time RT-PCR. The use of microarrays for the analysis of gene expression has been gaining in popularity since it is a high-throughput method to simultaneously evaluate a large number of genes for altered expression. However, due to the potential for falsely identifying altered genes by the microarray, it is recommended that data are validated by another means such as quantitative real-time RT-PCR (154, 191). We determined the immunologic perturbations that occurred in the MLN of BALB/c mice after both acute and chronic infections, as well as in the MLN and jejunums of C57BL/6 mice after a chronic

infection. Microarrays were performed during acute infections since cytokines of the innate immune system, such as type I interferons, are rapidly induced after viral infections (13). Chronic MNV infections were evaluated to assess the effect of viral persistence and chronic antigen-host interaction on the immune response. The MLN was chosen for analysis since it is associated with lymphatic drainage and immunologic sampling of the small intestines (23). The jejunum was evaluated since both the MLN and jejunum are sites of persistent MNV infections in mice (85) and presumably interactions between the virus and immune cells. The mouse strains selected for this study are two commonly used strains in research and they encompass both resistant (C57BL/6) and susceptible (BALB/c) mouse strains to various microbial infections (19, 80, 107, 112). These traits of resistance and susceptibility to microbial infections may be related to their T helper cell (Th) responses since in some infectious disease studies, C57BL/6 mice generate a predominantly Th1 response associated with cell-mediated immunity while BALB/c mice generate a predominantly Th2 response associated with humoral immunity (55, 80, 83, 107, 113).

Initially, multiple genes associated with the inflammatory response were identified by the microarray to have increased gene expression in the MLN of MNV infected BALB/c mice at various time points PI (Table 5.2) while only 1 gene was altered in the jejunum of C57BL/6 mice at week 4 PI (Table 5.3). However, of the subset of genes tested by quantitative real-time RT-PCR, none could be confirmed as having altered expression. Although increased expression of CXCL10 in MNV infected BALB/c mice was found at day 3 PI by both microarray and real-time RT-PCR, the fold change in expression by real-time RT-PCR (1.48-fold) was lower than our defined

threshold of a 2-fold change to conclude biological significance. Using a threshold of greater than a 2-fold change in expression has been previously reported in the literature to exhibit a greater correlation between microarray and real-time RT-PCR results (154, 190, 191). Additionally, the 1.48-fold change in expression of CXCL10 found by real-time RT-PCR was less than that determined by microarray analysis (3.13-fold), making the putative changes in CXCL10 expression less convincing since real-time RT-PCR is considered more sensitive than microarrays (180, 224). Therefore, it would be expected that the fold change in expression measured by real-time RT-PCR would be greater than that determined by the microarray and not lower as we found in this study. Furthermore, although some of the additional genes tested by quantitative real-time RT-PCR for altered expression had statistically significant changes by the Student's t-test (IFN-γ, IL-1β, and CCR1), they had very small fold-changes in expression (0.77 to 1.32) suggesting that they may not be biologically significant. However, it would be of value to confirm this hypothesis by measuring the protein expression of CXCL10, IFN-γ, IL-1β, and CCR1 in MNV infected mice to determine if these minor changes in mRNA expression are associated with detectable changes in protein expression.

In light of the difficulties confirming microarray findings to determine inflammatory gene changes that occur in MNV infected mice, quantitative real-time RT-PCR was performed on a selected group of genes reported to be altered in a number of other viral infections (18, 53, 107, 132, 148, 152, 175). Of these additional genes tested, the type I interferons, IFN-α and IFN-β, had increased gene expression in the MLN of MNV infected BALB/c mice that were both statistically significant by the Student's t-test and had a greater than 2-fold change at day 3 and week 4 PI (Figure 5.2). Likewise,

recent reports have described the importance of interferon receptors and interferon signaling pathways to prevent lethal disease in MNV infected STAT1 or IFNαβγ receptor knockout mice (106, 157). Therefore, to confirm the changes in expression of IFN-α and IFN-β found by our quantitative real-time RT-PCR assay in this study, ELISAs were performed to measure serum protein levels of IFN-α and IFN-β in MNV infected mice at day 1 and day 3 PI. However, no IFN protein expression could be detected in the serum at either day 1 or day 3 PI in MNV-4 infected mice and so assays for week 4 PI sera were not performed. It is unclear why no type I interferon proteins were detected in the serum of MNV infected mice in this study considering our positive quantitative real-time RT-PCR results at day 3 PI and a previous report indicating increased serum IFN-β at 24 hours PI in MNV-1 infected strain 129 mice (157). We speculate that these discrepancies may be due to a variety of reasons. First, the absolute copy numbers of IFN-α and IFN-β mRNA determined by quantitative real-time RT-PCR in our study was quite low, typically ranging from 5 to less than 100 copies with crossing points between 30 and 35 cycles. At these low copy numbers and high cycle thresholds, false elevations in fold-change of gene expression may be more evident since the variability due to assay performance from run to run becomes more pronounced. For example, a change in expression from 5 copies of mRNA to 20 copies of mRNA is quite large in terms of fold change, but most likely due to assay variability and not to a biologically significant increase in gene expression. A second reason that may account for the lack of detection of type I interferons in MNV infected mice is that our study used MNV-4 and BALB/c mice to measure serum proteins while the previous report found elevations of serum and jejunal IFN-β in MNV-1 infected strain 129 mice (157). Therefore, the norovirus strain

or mouse strain used in this study may be a contributing factor to the differences in expression of type I interferons. It has been previously shown that MNV-1 is rapidly cleared by the mouse immune system while MNV-4 causes persistent infections in immunocompetent mice (85, 106, 157), exemplifying that biological differences exist among viral strains. Also, previous studies have supported the idea that the inflammatory response to various inciting causes can be influenced by mouse strain and genetics (161, 162). A third reason that may explain our findings is that in the previous report using MNV-1 infected strain 129 mice, they revealed a peak of serum IFN-β at 24 hours PI with baseline levels at 12 and 72 hours PI (157). Since there is a short window of time to detect these increases of type I interferons, we may have temporally missed the IFN peak in our study. A fourth reason type I interferons were not detected may be that MNV-4 induces only a localized interferon response in the jejunum and not a systemic response detectable in the serum. However, in dispute of this, MNV-4 causes persistent infections in various tissues (85) so it was anticipated that a systemic inflammatory response that was detectable in the serum would occur in infected mice. Finally, perhaps the interferon was degraded in our serum sample or the level of expression in the serum was below the limit of detection of the ELISAs. However, these are unlikely since serum was rapidly processed and frozen soon after collection according to recommended protocols and the previous report using MNV-1 found elevations of serum IFN-β greater than 1000 pg/ml (157), which is well above the assay limit of 12.5 pg/ml and 15.6 pg/ml for IFN-α and IFN-β, respectively. Consequently, it would be interesting to perform additional experiments using MNV-1 and strain 129 mice to confirm the previous report of increased serum IFN-β levels. Also, it would be of value to infect BALB/c mice with

MNV-1 to evaluate if this norovirus strain will also induce a detectable serum type I interferon response in these mice, possibly contributing to its clearance, which was not seen with MNV-4.

Interestingly, at day 1 PI, none of the MLNs in MNV inoculated BALB/c mice and only 7 of 16 MLNs at day 3 PI were positive for MNV RNA by RT-PCR, even though 3 days was assumed to be more than ample time for the virus to traffic to the MLN. In strain 129 mice infected with MNV-1, a viral strain that does not cause persistent infections, viral dissemination to the spleen and liver were detectable by day 1 PI and to the lungs by day 3 PI (157). Our results were thus unexpected and were considered a result of insufficient time for virus trafficking, perhaps due to differences in viral strain or mouse strain used in this study, rather than evidence of unsuccessful MNV infection in these mice. In support of this, and similar to previous reports (85, 213), all mice evaluated at week 4 PI were positive for MNV by RT-PCR indicating that all mice become persistently infected after oral inoculation with MNV. Alternatively, the virus may have been present in low levels in the MLN at day 1 and day 3 PI but was below the limit of detection of our RT-PCR assay. However, in spite of the lack of detectable MNV RNA in the MLN, it was hypothesized that immunologic responses and signaling would still be occurring in the MLN of these mice that could be detected by microarray or quantitative real-time RT-PCR. As an additional measure to evaluate if the presence or absence of MNV RNA in the MLN influenced the experimental data, gene expression data from the microarray and quantitative real-time RT-PCR at day 3 PI were segregated and evaluated taking into consideration the positive or negative MNV RNA results and no significant changes in data interpretation were noted (data not shown).

The questions therefore remain, what are the immunologic perturbations that occur in laboratory mice infected with MNV, and do these perturbations contribute to confounding biomedical research using MNV infected mice? There is the possibility that the studies reporting herein define the actual response to MNV infection in laboratory mice; that no significant alterations in the immune system occur after infection. In support of this, MNV infection does not induce any overt clinical disease or histopathologic lesions in immunocompetent, neonatal, or many strains of severely immunocompromised mice such as those lacking T and B cells (84, 85, 106, 156, 157, 213, 225), indicating that MNV is well adapted to the host. Thus, could MNV be considered a commensal organism, even though this categorization is typically reserved for bacterial agents and not viral infections (69)? Or was the experimental design, genes, time points, tissues, viral strain, mouse strain, or assays used in this study to detect immunologic changes not optimal and require refinements? Based on previous reports indicating the importance of the innate immune system in preventing lethal MNV infection, the robust antibody response that occurs in infected immunocompetent mice, and the chronic antigenic stimulation that presumably occurs due to persistent MNV infections (84, 85, 106, 157, 213, 230), these findings would argue that an immunologic response is indeed occurring in these mice that we were unable to identify in this study, rather than the lack of an immune response to MNV altogether. Furthermore, even when using two different mouse strains considered susceptible (BALB/c) and resistant (C57BL/6) to various microbial infections, we were unable to make any conclusions about differences in susceptibility to MNV since both strains of mice became persistently infected and did not show any signs of disease or exhibit any detectable changes in

inflammatory gene expression. Additional experiments infecting additional strains of mice with MNV would be worthwhile to help determine if genetic factors contribute to the immunologic response. In vitro assays could also be performed with various strains of MNV to elucidate differences in expression of inflammatory cytokines and chemokines after infection. All of these studies would potentially provide valuable information on the pathogenesis of MNV infection and the potential impact of MNV in laboratory mice used for biomedical research.

CHAPTER VI

Serologic Evidence of a Rat Norovirus

INTRODUCTION

Infection with members of the genus *Norovirus* have been found in many species including humans, cattle, swine and mice (48, 63, 73, 106, 174, 221). In humans, noroviruses are the major cause of epidemic nonbacterial gastroenteritis worldwide (45, 46, 63). Clinical signs typically consist of severe vomiting and diarrhea lasting for 24 to 48 hours which spreads rapidly from person to person (57, 93). Norovirus infections in cattle has been identified by electron microscopy and reverse transcriptase-polymerase chain reaction (RT-PCR) from fecal samples of both clinically normal and diarrheic calves (73, 128, 202, 217). In pigs, norovirus infection has been detected in asymptomatic animals and its role in causing diarrhea is still being elucidated (208, 217, 221, 223). The first report of a norovirus infecting mice, designated murine norovirus (MNV), was only recently described in 2003 causing lethal infections in specific strains of immunodeficient laboratory mice (106). However, in immunocompetent mice and some immunodeficient strains not susceptible to lethality, norovirus infection does not cause any overt clinical signs of disease (84, 85, 106). Rather, these mice are persistently infected and asymptomatically shed infectious virus in the feces for prolonged periods (85, 106).

Testing by our diagnostic laboratory of sera from laboratory mice used in biomedical research by a high-throughput serologic assay, the multiplex fluorescent immunoassay (MFI), revealed that 22.1% of 12,639 serum samples contained antibodies to MNV (84). These samples were submitted from institutions throughout the United States and Canada, indicating widespread infection in research mice. MNV has also been reported in Germany indicating its worldwide distribution (156, 213). Therefore, the high serologic prevalence of MNV infection in laboratory mice from widespread geographical locations suggests that these noroviruses have been present for quite some time and were only recently recognized, rather than the sudden emergence of a novel norovirus in a new host species with rapid worldwide dissemination. The only recent discovery of MNV in laboratory mice may be due, in part, to the lack of clinical signs or disease caused by MNV that could be used to identify infected laboratory mice and the previous lack of diagnostic assays to detect MNV infection in mice. These characteristics, in concert with their capability to cause persistent infections and prolonged fecal shedding of infectious virus, may have played a significant role in the transmission and maintenance of MNV in laboratory mice prior to its discovery.

Therefore, based on the recent discovery of a norovirus in mice, as well as recognizing that noroviruses infect many different host species, we hypothesized that a yet to be discovered norovirus in rats may exist. In order to isolate a novel rat norovirus, serologic assays using MNV as the antigen were performed to identify suspected norovirus infected colonies of laboratory rats. Serologic cross-reactivity among noroviruses infecting different host species has been previously reported between swine and humans (48), and cross-reactive antibodies to human noroviruses have been

documented in a number of non-human primate species (94). In addition, molecular and cell culture techniques on tissue samples from seropositive rats were utilized in attempts to obtain nucleotide sequence data and a cultivatable strain of a novel rat norovirus. Herein this report, we show serologic evidence of norovirus antibodies in rat sera that cross-react with antigens from a mouse norovirus. However, both molecular and cell culture techniques were unsuccessful in obtaining either nucleotide sequence or a cultivatable isolate of a novel rat norovirus.

MATERIALS AND METHODS

Animals and sample collection. Samples were collected from rats that were received by the Research Animal Diagnostic Laboratory from a variety of sources including commercial vendors, research institutions, and pet stores. The majority of rats were animals used in health monitoring programs and consisted of adult female SD rats, however for some animals, information was not provided. Blood was collected by cardiocentesis from rats that were euthanized with an overdose of inhaled carbon dioxide. The blood was allowed to clot, the serum separated by centrifugation at 2000 × g, and stored at -20°C until use. Mesenteric lymph node, spleen and fecal samples were collected from rats at necropsy using aseptic technique and stored at -80°C until use.

Microsphere fluorescent immunoassay (MFI). The MFI was performed as previously described (84, 85) with some modifications. Modifications included using MNV-4 proteins as the antigen, trichlorotrifluoroethane (Fisher Scientific, Fair Lawn, NJ) instead of 1% Nonidet P-40 substitute during concentration and purification of viral proteins, and a phycoerythrin conjugated $F(ab')_2$ fragment goat anti-rat IgG (H+L)

(Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) as the secondary detector antibody. Test serum samples were assayed at a final serum concentration of 1:500 in a 100µl test volume.

Western blot. Western blots were performed as previously described (84) with some modifications. Modifications included using 25µg of cesium chloride-purified MNV-4 proteins and a biotinylated goat anti-rat IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Indirect fluorescent antibody (IFA) assay. IFA assays were performed as previously described (84) with some modifications. Modifications included infecting RAW 264.7 cells with MNV-4 and using goat anti-rat IgG (H+L) fluorescein isothiocyanate (FITC)-labeled secondary antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

RNA extraction and RT-PCR. Tissue and fecal samples were homogenized as previously described (84) and RNA was extracted using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) or the MagAttract RNA Tissue Mini M48 Kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommended protocol. RNA was extracted from clarified cell culture supernatants using the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommended protocol. RT-PCR was performed using the OneStep RT-PCR Kit without Q-Solution (Qiagen, Valencia, CA) according to manufacturer's recommended protocol and thermocycling parameters consisting of reverse transcription at 50ºC for 30 min; activation of DNA polymerase at 95ºC for 15 minutes; 40 cycles of denaturation (94ºC, 30 sec), annealing (48ºC, 30 sec), and extension (72ºC, 30 sec); and a final extension at 72ºC for 10 min. RT-PCR products

were separated on precast 3% agarose gels containing ethidium bromide (Bio-Rad Laboratories, Hercules, CA) and visualized under ultraviolet light. Amplicons were purified from the agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA) and nucleotide sequencing performed by SeqWright DNA Technology Services (Houston, TX).

RT-PCR primer pairs were designed to bind within conserved regions based on alignments of multiple norovirus genomes infecting various host species using the Discover Studio Gene software package version 1.5 (Accelrys, San Diego, CA). Nucleotide sequences that were aligned and used for primer pair selection were from noroviruses infecting mice (MNV-1, AY228235.2; MNV-2, DQ223041; MNV-3, DQ223042; MNV-4, DQ223043), cattle (Jena, AJ011099), and humans (Norwalk, M87661; Southampton, L07418). In addition, previously reported primers that were described to bind within conserved regions of norovirus genomes were also used. Primer pairs are listed in Table 6.1.

Cell culture. Mesenteric lymph nodes collected aseptically from rats were pooled and added to 5 mL of Dulbecco's modified Eagle's medium (HyClone, Logan, Utah) supplemented with 10% low-endotoxin fetal bovine serum (Cambrex, East Rutherford, N.J.), and 10 μg/ml of ciprofloxacin (supplemented growth medium). Samples were then processed in a blender (Stomacher 80 Lab Blender, Tekmar Company, Cincinnati, Ohio) for 1 min and clarified by centrifugation for 10 min at 10,000 × g. RAW 264.7 cells, a mouse macrophage cell line, and NR8383 cells, a rat macrophage cell line, were inoculated with 1 to 2 mL of clarified mesenteric lymph node homogenate in 25 cm^2 cell culture flasks and incubated for 1 hour at 37°C and 5% CO_2,

Table 6.1. Primers used to amplify a novel rat norovirus.

Primer Name	Primer sequence (5' to 3')		Amplicon size	Reference
	Forward	Reverse		
Rat Noro 1	CAYGCCAACAACAAYATGTAT	TCACATAAKGGSCCAAARGC	176	
Rat Noro 2	AAYMMNATTGAYCCCTGGAT	CCAACCCARCCRKTRTACAT	167	
Rat Noro 3	WWGGTGARTTTWCYATTTC	WGTRCGGAGTGGSGTGTA	386	
MNV internal 1F/3R	CACATGCTTCCCACATGTC	ACCAGGCGCATAGATTCCT	123	
MNV C	CAGATCACATGCTTCCCAC	AGACCACAAAAGACTCATCAC	188	(85)
P289/P290	TGACAATGTAATCATCACCATA	GATTACTCCAAGTGGGACTCCAC	319	(98)
P110/P290	ACDATYTCATCATCACCATA	GATTACTCCAAGTGGGACTCCAC	317	(123, 221)

after which the inoculum was replaced with fresh supplemented growth medium and incubated at 37°C with 5% CO_2. Cultures were monitored visually for cytopathic effect as an indicator of viral infection and propagation. If no cytopathic effect was observed after 7 days, cultures were passaged by freezing and thawing the cells for 3 cycles, centrifuging at 10,000 × g, and inoculating fresh cell cultures as described above.

RESULTS

Serologic evidence of a rat norovirus. To determine if a host-specific norovirus existed in laboratory rats, the high-throughput MFI assay was used to screen a large number of rat serum samples received by our diagnostic laboratory from April 11th to September 20th, 2006 for antibodies that cross-reacted with MNV antigens. Of the approximately 5000 serum samples tested, 18 samples had intermediate (between 175 and 600) or positive (>600) MFI fluorescence values based on thresholds previously defined for testing mouse sera (84). Of these 18 samples, 11 (61.1%) were positive by the MNV IFA assay characterized by visualization of a cytoplasmic fluorescence pattern typical of RNA viruses (Figure 6.1A). The remaining 7 serum samples were either negative or sufficient serum was not available for further analysis. Two serum samples with positive MFI fluorescence values (786 and 1288) that were also positive by the IFA assay were tested by the MNV Western blot assay. The Western blot assay revealed a specific band at approximately 59 kDa (Figure 6.1B, lanes 3 and 4) representing the capsid protein as previously reported for noroviruses (84, 106). These bands aligned with a band of similar size produced when the same blot was probed with serum from a mouse experimentally infected with MNV-4 (Figure 6.1B, lane 7).

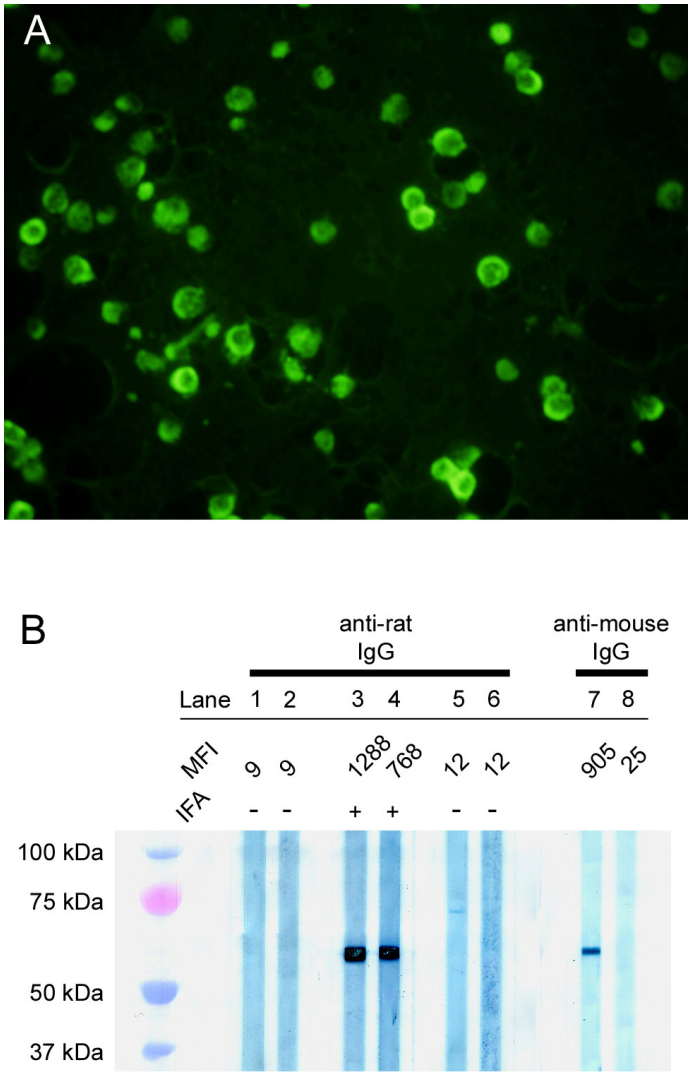

Figure 6.1. Serologic assay results of rat serum samples tested for norovirus antibodies using MNV-4 as the antigen. A) A positive IFA assay displaying cytoplasmic fluorescence when using MNV-4 infected RAW 264.7 cells probed with rat serum. B) Western blot assay probed with rat or mouse serum samples, and their corresponding MFI and IFA results. A band at approximately 59 kDa in size representing the capsid protein was displayed when probed with rat (lanes 3 and 4) or mouse (lane 7) serum samples.

Of the rat serum samples that were positive by the MNV serologic assays, a large proportion were obtained from a single colony of rats used in research. Therefore, continued screening of rats from this colony for 6 months after the initial positive results identified additional serum samples with evidence of antibodies to a rat norovirus. These serum samples were positive by both IFA and Western blot assays, and had MFI fluorescence values that were intermediate or negative based on thresholds defined to test mouse sera (Figure 6.2, lanes 2 and 3). Additionally, some serum samples were positive by the MNV IFA assay but were negative by both the Western blot assay and MFI assay (Figure 6.2, lanes 1 and 5).

RT-PCR of tissues and fecal samples. In attempts to obtain the nucleotide sequence of a novel rat norovirus, tissue and fecal samples were collected from rats that either had serologic evidence of cross-reactive antibodies to MNV based on the MFI, IFA and/or Western blot assays, from rats that had evidence of infection with other pathogens such as *Helicobacter* sp., pinworms, parvoviruses, and rat theilovirus, or from rats obtained from pet stores. RNA extracted from the mesenteric lymph nodes, spleen, and feces was tested by RT-PCR with primers designed or reported to bind to conserved regions of norovirus genomes from multiple different host species. Designed primer sets were initially tested using MNV-4 RNA and all primer sets produced amplicons of the expected size (Figure 6.3). Amplicons produced from RT-PCR of RNA extracted from rat tissues and feces that were approximately the expected product size were purified and sequenced. None of the nucleotide sequences obtained significantly aligned with nucleotide sequences of known noroviruses, but rather aligned with murine genomic sequences.

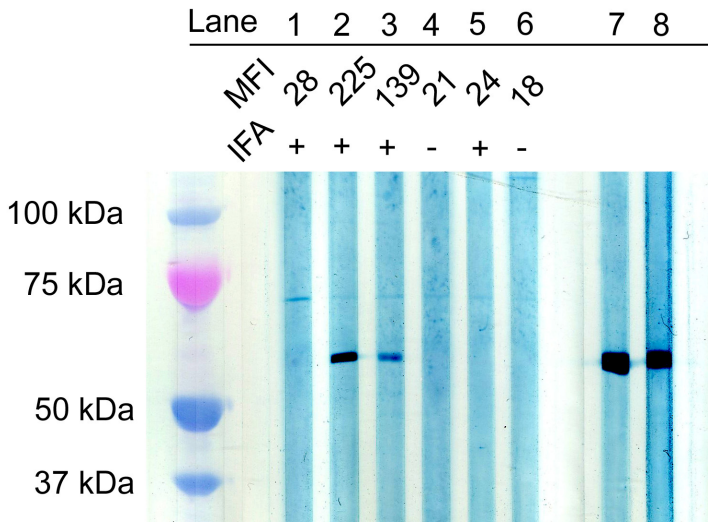

Figure 6.2. Western blot analysis of rat serum samples obtained from a group of rats housed in a research colony suspected of having an endemic rat norovirus infection. Corresponding MFI and IFA results are also show. Serum samples with positive IFA and Western blot assay results had either intermediate (lane 2) or negative (lane 3) MFI fluorescence values based on previously defined thresholds for testing mouse sera (84). Additionally, some serum samples had positive IFA assay results but were negative by both MFI and Western blot assays (lanes 1 and 5). Lanes 7 and 8 were positive controls and were probed using the same rat serum samples show in Figure 6.1B, lanes 3 and 4.

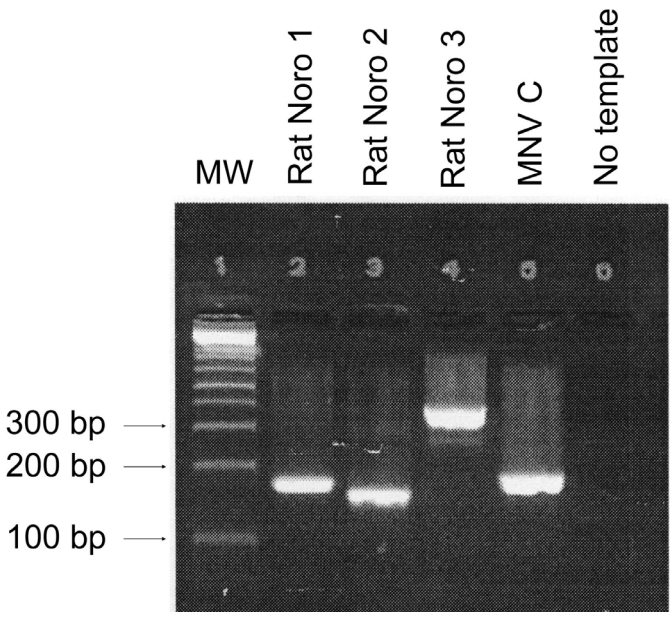

Figure 6.3. Ethidium bromide-stained agarose gel of specific RT-PCR products of expected size when using primer pairs designed to bind within conserved regions of norovirus genomes infecting different host-species (lanes 2-4). Template RNA was extracted from cesium chloride-purified MNV-4. The MNV C primer pair (lane 5) was used as a positive control.

Cell culture for a novel rat norovirus. In attempts to obtain a cultivatable rat norovirus, mesenteric lymph nodes from rats with serologic evidence of norovirus antibodies by the MFI, IFA and Western blot assay were homogenized, clarified, and inoculated onto a mouse macrophage cell line (RAW 264.7 cells) or a rat macrophage cell line (NR8383 cells). Cells were maintained for 5 passages and evaluated for cytopathic effect suggestive of norovirus infection. No evidence of cytopathic effect was seen in either cell line in any of the 5 passages. In addition, IFA slides were produced from passage 5 of inoculated RAW 264.7 cells and NR8383 cells and probed with both rat and mouse sera containing norovirus antibodies. No specific fluorescence was noted in any of the IFA assays. Finally, RT-PCR was performed with the primers listed in Table 6.1 on RNA extracted from the clarified supernatants of mesenteric lymph node homogenates used to inoculate the first passage of cell cultures. However, no norovirus specific nucleotide sequences were obtained.

DISCUSSION

Noroviruses are within the family *Caliciviridae* which includes viruses that infect a wide range of host species such as humans, cattle, pigs, cats, dogs, rabbits, sea lions, reptiles, amphibians, fish, birds and mice (6, 63, 73, 106, 149, 221). Since a norovirus infecting mice was only recently recognized in 2003 and subsequently shown to infect a significant proportion of laboratory mice used in biomedical research (84, 106), we hypothesized that a host-specific norovirus may exist in laboratory rats. To pursue this hypothesis, the MFI, a high-throughput serologic assay, was used to screen a large number of rat serum samples submitted to our diagnostic laboratory to try and identify

rats that had serologic evidence of a rat norovirus. In this study, we used a mouse specific norovirus, MNV-4, as the antigen in the MFI since serologic cross-reactivity among noroviruses infecting different host species has been previously reported (48, 85). Initially, two serum samples from sentinel rats with positive MFI values were also positive when tested by IFA and Western blot assays. Notably, the Western blot assay revealed a band at approximately 59 kDa, the size of the major antigenic capsid protein (106), that also aligned with a band produced when the same Western blot membrane was probed with a serum sample from a mouse experimentally inoculated with MNV. These serologic results suggest that there is a norovirus in laboratory rats capable of producing a productive infection and seroconversion in these rats. These results were corroborated when additional positive serum samples were identified 6 months later from a new group of sentinel rats housed in the same colony as the initial two positive rats. These findings suggest that an endemic infection with a rat norovirus existed in this colony since typically the purpose of sentinel animals is to provide a representation of infectious agents in a colony that can be transmitted by soiled bedding to naïve sentinels. Interestingly, a few serum samples that were considered positive by the IFA assay were negative when tested by Western blot assays and MFI. The most likely reason to account for this discrepancy is that the IFA assay is dependent upon subjective interpretation by the observer (111), and therefore these results are most likely false positives. Alternatively, since a heterologous mouse norovirus antigen was used in our serologic assays rather than a homologous rat norovirus antigen, it is possible that the sensitivity, specificity, or thresholds of the serologic assays were less than optimal.

Attempts to obtain a cultivatable rat norovirus by cell culture of mesenteric lymph node homogenates was unsuccessful when using either a mouse or rat macrophage cell line, even though MNV has been described to efficiently replicate in mouse macrophages and dendritic cells (230). Historically, norovirus research has been hampered by the lack of a cell culture system (42). Therefore, our results are not unexpected since successful cultivation of noroviruses has only recently been described in a limited number of reports (7, 109, 206, 230). Our attempts to obtain nucleotide sequence data of a novel rat norovirus by RT-PCR were also unsuccessful, even when nucleotide alignments of noroviruses infecting different host-species were used to find conserved regions of the genome for primer design. These results are not unexpected since genetic similarity among the full-length nucleotide sequences of noroviruses can be as low as 52.3% between noroviruses infecting different host species, and even as low as 55.6% between human strains (86). Also, it is unknown whether norovirus infections in rats are persistent such as in the mouse (85), or whether they are transient such as in humans (63). Therefore, selecting rats using serologic assays that test for the presence of norovirus specific antibodies may not be optimal for in vitro cultivation or RT-PCR analysis since rats may have produced an effective immune response and cleared viral infection by the time antibody is detectable.

Even though a mouse specific norovirus was used as the antigen to document serologic evidence of a rat norovirus, we speculate that these results are indeed suggestive of a novel host-specific rat norovirus producing cross-reactive antibodies in rats, and not due to a rat becoming infected with a mouse specific norovirus. The reasons for this hypothesis are: (i) noroviruses are considered to be highly host-specific and

attempts to infect and induce disease with human noroviruses in a wide range of species including non-human primates, mice, cats, guinea pigs, calves and rabbits have been unsuccessful (36, 63, 197, 207), (ii) serologic cross reactivity among noroviruses infecting different host species has been previously reported to occur (48), and (iii) experimental infection studies of male SD rats inoculated by oral gavage with 1×10^7 plaque forming units of MNV-4 at 5-weeks-old failed to induce a detectable antibody response when measure by MFI 4-weeks after inoculation suggesting infection was unsuccessful (R.T. Stoffel, L.K. Riley, and R.S. Livingston, unpublished data).

In conclusion, serologic evidence of antibodies to a norovirus in rats was documented by MFI, IFA and Western blot assays. Although we could not obtain a cultivatable rat norovirus or identify any nucleotide sequence data, additional testing is warranted to confirm the presence of a potentially undiscovered norovirus in laboratory rats.

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VITA

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