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Author manuscript

Science. Author manuscript; available in PMC 2015 July 16.

Published in final edited form as:

Science. 2015 January 16; 347(6219): 266–269. doi:10.1126/science.1258025.

Commensal microbes and interferon-λ determine persistence of enteric murine norovirus infection

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Abstract

The capacity of human norovirus (NoV), which causes >90% of global epidemic nonbacterial gastroenteritis, to infect a subset of people persistently may contribute to its spread. How such enteric viruses establish persistent infections is not well understood. We found that antibiotics prevented persistent murine norovirus (MNoV) infection, an effect that was reversed by replenishment of the bacterial microbiota. Antibiotics did not prevent tissue infection or affect systemic viral replication but acted specifically in the intestine. The receptor for the antiviral cytokine interferon-λ, *Ifnlr1*, as well as the transcription factors *Stat1* and *Irf3*, were required for antibiotics to prevent viral persistence. Thus, the bacterial microbiome fosters enteric viral persistence in a manner counteracted by specific components of the innate immune system.

The microbiota of the intestine, including bacteria, fungi, viruses, and the meiofauna, contributes to both enteric disease and homeostatic immune function (1-7). Given the ability of human norovirus (NoV) to establish persistent infections in people (8-10), it is important to identify mechanisms of enteric NoV persistence. This task is made possible by the identification and molecular cloning of murine NoV (MNoV) strains that are capable of only acute enteric infection (strain CW3) or both acute and persistent infection (strain CR6) (11-14).

Because bacteria can interact with viruses in the intestine to alter intestinal physiology and cause pathology (2, 6, 15, 16), we tested the hypothesis that bacteria influence persistent enteric infection *in vivo* by treating C57BL/6J mice (control mice herein) for 2 weeks with broad-spectrum oral antibiotics (vancomycin, neomycin, ampicillin, and metronidazole; Abx or antibiotics herein), then inoculating them orally with 10⁶ plaque-forming units (pfu) of MNoV CR6. Antibiotics prevented persistent enteric infection in the majority of animals as measured by fecal viral shedding (**Fig. 1, A and B**) and by levels of virus in intestinal tissues 3 and 14 days, after inoculation (**Fig. 1, C and D**) but had no direct inhibitory effect

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on virus replication in cultured cells (**fig. S1, A and B**). The source of differential MNoV levels at 4 and 24 hours (**Fig. 1A**) is the delayed intestinal transit time of the viral inoculum in Abx-treated mice (**fig. S2**). We confirmed the durability and efficacy of antibiotic effects by the lack of viral shedding and diminished tissue infection 35 days after inoculation (**fig. S3, A and B**) and reduced antiviral antibody response, which correlated with reduced virus levels (**fig. S3C**).

Viral persistence could not be consistently prevented with a single antibiotic targeting different bacterial species (**fig. S4**). In comparison, vancomycin plus ampicillin had an effect similar to that of the antibiotic cocktail (**fig. S4**). Successful inhibition of viral persistence in general correlated with a substantial reduction in detectable 16S ribosomal DNA copies (**Fig. 2A**). In some cases, antibiotic treatment did not decrease 16S copies, but this did not correlate with a failure to prevent MNoV persistence (**fig. S5**). To confirm the importance of bacteria, we replaced antibiotics with drinking water for 3 days prior to CR6 infection to allow for drug clearance. Mice were still resistant to CR6 infection in this setting (**Fig. 2, B and C**). We then performed fecal transplantation to replace the intestinal microbiota. Fecal transplantation from control mice was sufficient to rescue the ability of CR6 to infect mice persistently, as measured by both fecal shedding and establishment of infection in intestinal tissues (**Fig. 2, B and C**). However, fecal transplants from antibiotic-treated mice did not rescue CR6 infection (**Fig. 2, B and C**). These findings strongly implicate the intestinal microbiota in the establishment of persistent enteric NoV infection.

It was possible that the effects of antibiotics were specific to the intestine, the only tissue in which persistent CR6 infection is supported in control mice (12). To measure the effect of antibiotics on systemic viral infection, we used three approaches. First, we inoculated CR6 by intraperitoneal injection to allow systemic spread (**fig. S6, A and B**), a method that did not consistently lead to persistent infection (**fig. S6B**). Antibiotic treatment prior to intraperitoneal infection with CR6 did not alter viral levels at day 3 (**fig. S6A**). Second, we infected *Ifnar1^{-/-}* mice, which lack the gene that encodes the α chain of the interferon (IFN) α and β receptor and so are unable to respond to type I IFNs, and in which CR6 spread to and replicated persistently in the mesenteric lymph node (MLN) and spleen (17) (**Fig. 3A**). Levels of MNoV RNA in the MLN, the draining lymph node for the intestine, likely reflect both systemic and intestinal infection. Antibiotics prevented intestinal replication in these mice but had no effect on replication in the MLN and spleen (**Fig. 3A**). Third, we used a genetic approach to determine whether antibiotics acted specifically in intestinal tissues. MNoV strain CW3 spreads to systemic tissues after oral inoculation, a property conferred by the CW3 capsid protein VP1 (12, 18). We therefore tested the effects of antibiotics on infection with a chimeric virus with the CR6 backbone and VP1 of CW3 (CR6^{VP1-CW3}), which effectively replicates in both intestinal and systemic tissues (12, 18). Antibiotics had no effect on acute CW3 or CR6^{VP1-CW3} replication in the spleen and MLN after oral inoculation. This indicated that antibiotics did not prevent viral entry into host tissues. However, antibiotics prevented replication of both CR6 and CR6^{VP1-CW3} in the intestine at 3 and 14 days (**Fig. 3, B and C**). Therefore, the effects of antibiotic treatment are specific to the intestine.

To test further whether antibiotics inhibit viral trafficking from the lumen into tissues, we examined viral levels in Peyer's patches (PP), which have been identified as the site of MNoV entry via M cells (19). Virus was detectable in PP at equivalent levels in antibiotic-treated and control mice 1 and 3 days after infection (**Fig. 3D and fig. S7**), although by day 3, antibiotic treatment substantially lowered viral levels in the ileum and colon. We conclude that virus successfully trafficked from the intestinal lumen to the mucosa via PP even in the presence of antibiotics. Antibiotic-mediated prevention of persistent infection must therefore be via an effect of the bacterial microbiome on viral clearance from intestinal tissues.

Control mice infected with either 10^5 or 10^6 pfu of MNoV demonstrated antibiotic-mediated inhibition of infection, whereas a higher dose (10^7 pfu) overcame the effects of antibiotics (**Fig. 4A and fig. S8A**). This suggests that host pathways that regulate viral replication might play a role in antibiotic-mediated prevention of persistent enteric norovirus infection. To define the immunologic mechanism(s) responsible for antibiotic-mediated prevention of enteric persistence, we tested the involvement of candidate host pathways by treating mice carrying null mutations in specific immune genes with antibiotics followed by oral challenge with 10^6 pfu of CR6 (**table S1**). To address the well-established role of IFN signaling in antiviral host defense, we examined *Ifnar1^{-/-}*, *Ifngr1^{-/-}* (encodes IFN γ receptor 1), and *Irf7^{-/-}* (encodes the transcription factor IFN regulatory factor 7, which is required for the IFN- α amplification loop) mice. To determine whether adaptive immunity or secretory immunoglobulin A (IgA), both previously implicated in NoV control (20, 21), was involved, we examined *Rag1^{-/-}* and *Pigr^{-/-}* mice lacking all T and B cell responses or the capacity to secrete IgA, respectively. Because lipopolysaccharide (LPS) signaling can increase virus infectivity (22-24), we assessed *Tlr4^{-/-}* mice lacking the innate immune receptor for LPS as well as mice deficient in the downstream signaling molecules Myd88 and Trif (encoded by *Ticam1*) required for Toll-like receptor signaling. We also examined mice lacking the genes that encode the bacterial sensor *Tlr2* and the double-stranded RNA recognition molecule *Mda5*, which is involved in MNoV triggering of type I IFN responses (25). Finally, because autophagy has been reported to contribute to epithelial cell defenses against enteric bacterial infection (26, 27) we examined *Atg16l1* HM mice, which are hypomorphic for expression of the autophagy protein Atg16L1 and which exhibit exacerbated intestinal pathology after CR6 infection (2). Remarkably, none of these genes were required for antibiotic-mediated prevention of persistent enteric MNoV infection, and indeed *Rag1^{-/-}* mice exhibited mild resistance to infection at baseline (**Fig. 4B and fig. S8B**)—a finding consistent with a role for B cells in MNoV infection (28).

Because IFN- λ signaling was reported to control acute intestinal rotavirus infection (29) and has a key role in controlling persistent MNoV infection (17), we examined the requirement for this pathway (30) in antibiotic suppression of persistent infection. *Ifnlr1^{-/-}* mice (lacking IFN- λ receptor 1), *Stat1^{-/-}* mice (lacking a key transcription factor that conveys IFN- $\alpha\beta$, IFN- γ , and IFN- λ signals), and *Irf3^{-/-}* mice (lacking the transcription factor IFN regulatory factor 3 that induces expression of IFN- $\alpha\beta$ and IFN- λ) (31, 32) were vulnerable to persistent CR6 infection even in the presence of antibiotics (**Fig. 4, B to D; fig. S8, A and B; and fig. S9, A to G**). Whereas antibiotic treatment prevented persistent infection of control mice inoculated with 10^5 pfu of MNoV (**Fig. 4A and fig. S8A**), some mice lacking components

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of the IFN- λ induction or signaling pathway became persistently infected even at this low dose (**Fig. 4D and fig. S8A**, $P < 0.01$). This vulnerability to MNoV infection did not correspond with differential effects of antibiotics on bacterial depletion in these mutant mice, as shown by 16S rDNA sequencing studies and by experiments in which fecal transplantation from antibiotic-treated mutant mice did not support establishment of persistent infection (**fig. S10, A to D**). Resistance to MNoV infection in antibiotic-treated control mice was not explained by up-regulation of *Ifnlr1* or a selected set of IFN-stimulated genes including *Isg15*, which has direct antiviral activity against MNoV (33) (**fig. S11, A to I**).

Our data suggest that the bacterial component of the enteric microbiome plays an essential role in controlling the capacity of a virus to establish persistent infection and that antibiotics can substantially alter the pathogenesis of enteric viral infection. Prior reports have shown that bacterial LPS can alter viral infectivity (22-24), but the effects of antibiotics reported here did not require TLR4 or other signaling molecules involved in host responses to LPS. Although the absence of B cells may explain the partial resistance to MNoV infection seen in *Rag1*^{-/-} mice (28), adaptive immunity was not required for antibiotic treatment to significantly inhibit persistent MNoV infection. Our results indicate that innate immunity and in particular the IFN- λ pathway, is required for the effects of antibiotic treatment on persistent infection. These observations suggest that the bacterial microbiota limits the efficacy of IFN- λ -dependent innate immunity or alters some yet-undefined innate immune pathway that renders viruses susceptible to the effects of IFN- λ . These data indicate that the clinical use of antibiotics in humans may alter the enteric virome (6, 34) and that the effects of antibiotics in the treatment of infectious diseases may not be entirely attributable to their antibacterial properties. Given the contribution of the virome to host physiology (4, 6), these data suggest the importance of considering the effects of trans-kingdom interactions for understanding the pathogenesis of infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. Liu for technical assistance, H. Lazear for discussions, D. Kreamalmeyer for animal care and breeding, members of the Virgin laboratory for manuscript review and discussion, and S. Doyle and Bristol-Myers Squibb for providing *Ifnlr1*^{-/-} mice. The mouse norovirus strains used in this paper are available from Washington University under a material transfer agreement. *Ifnlr1*^{-/-} mice were made available from ZymoGenetics Inc. (Bristol-Myers Squibb) under a material transfer agreement with Washington University School of Medicine. H.W.V. is a coinventor on a patent filed by Washington University School of Medicine related to the use of murine norovirus. The data presented in this manuscript are tabulated in the main paper and in the supplementary materials. Sequencing data have been uploaded to the European Nucleotide Archive (accession no. PRJEB7745). Supported by NIH grant R01 AI084887 and U19 AI109725, Crohn's and Colitis Foundation Genetics Initiative grants 274415, and Broad Foundation grant IBD-0357 (H.W.V.); NIH training grant 5T32CA009547 and the W. M. Keck Fellowship from Washington University (M.T.B.); NIH training grant 5T32AI007163 and postdoctoral fellowships from the Cancer Research Institute and American Cancer Society (T.J.N.); NIH grant 1F31CA177194 (B.T.M.); NIH training grant 5T32AI007163 (C.C.Y.); and NIH grants U19 AI083019 and U19 AI106772 (M.S.D.). Washington University and H.W.V. receive income based on licenses for MNoV technology.

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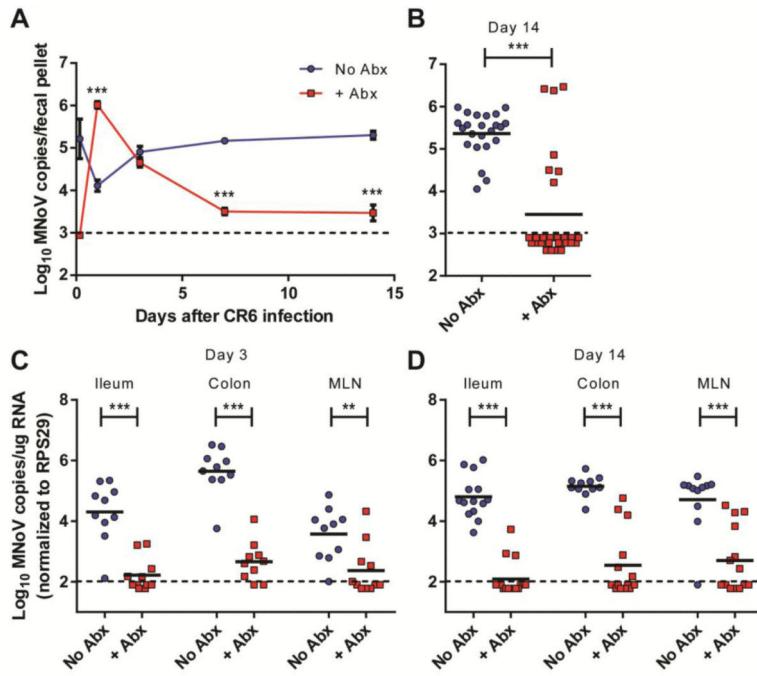


Fig. 1. Pretreatment with an antibiotic cocktail prevents establishment of persistent intestinal infection by murine norovirus strain CR6

Mice were treated with Abx (vancomycin, neomycin, ampicillin, and metronidazole) for two weeks prior to oral infection with 10^6 pfu of CR6. (A and B) Time course of MNoV genome copies shed into fecal pellets with time points at 4 hrs and 1, 3, 7, and 14 days with individual data points at day 14 in (B). Results analyzed by two-way analysis of variance (ANOVA) with Sidak's multiple-comparisons test. ANOVA $P < 0.0001$. $N = 7$ to 33 mice per cohort per time point combined from 3 independent experiments. (C and D) MNoV genome copies detected in ileum, colon or MLN at day 3 (C) or 14 (D) post-CR6 infection. Analyzed by Mann-Whitney test. $N = 10$ to 16 mice per cohort combined from three or four independent experiments. ** $p < 0.01$, *** $p < 0.001$.

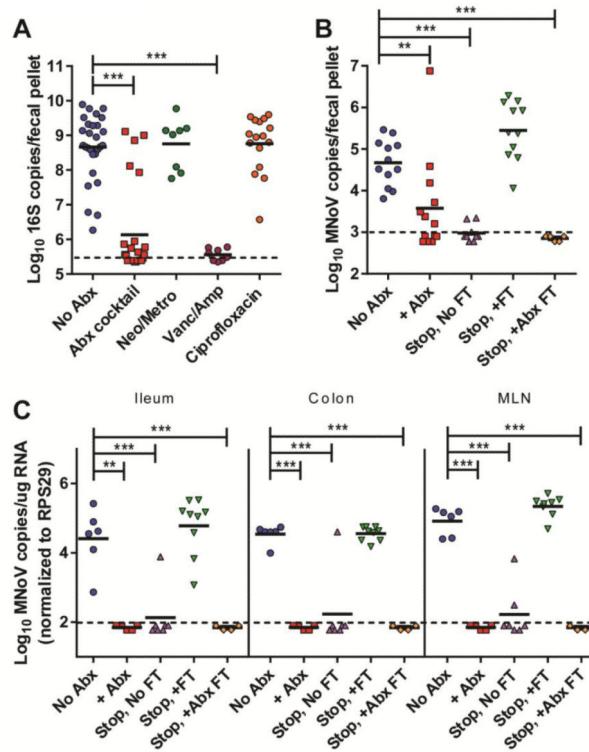


Fig. 2. Bacterial depletion prevents CR6 infection, and reconstitution of the intestinal microbiota rescues infection

(A) 16S rDNA copies per fecal pellet as detected by quantitative real-time polymerase chain reaction of the V4 hypervariable region of the 16S rRNA gene. Fecal pellets were collected after 2 weeks of the indicated antibiotic treatment. Neo, neomycin; Metro, metronidazole; Vane, vancomycin; Amp, ampicillin. ANOVA $P < 0.0001$; $N = 8$ to 22 mice per cohort combined from two to five independent experiments. (B and C) Mice received no Abx, continuous Abx treatment (+Abx), or discontinuance of Abx with no fecal transplantation (Stop, No Ff), transplantation from control untreated mice (Stop, +Ff), or transplantation from control Abx-treated mice (Stop, +Abx Ff). MNMV genome copies were detected at day 14 in fecal pellets (B) or indicated tissues (C). ANOVA $P < 0.0001$; $N = 4$ to 9 mice per cohort combined from three independent experiments. All data were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test. ** $P < 0.01$, *** $P < 0.001$.

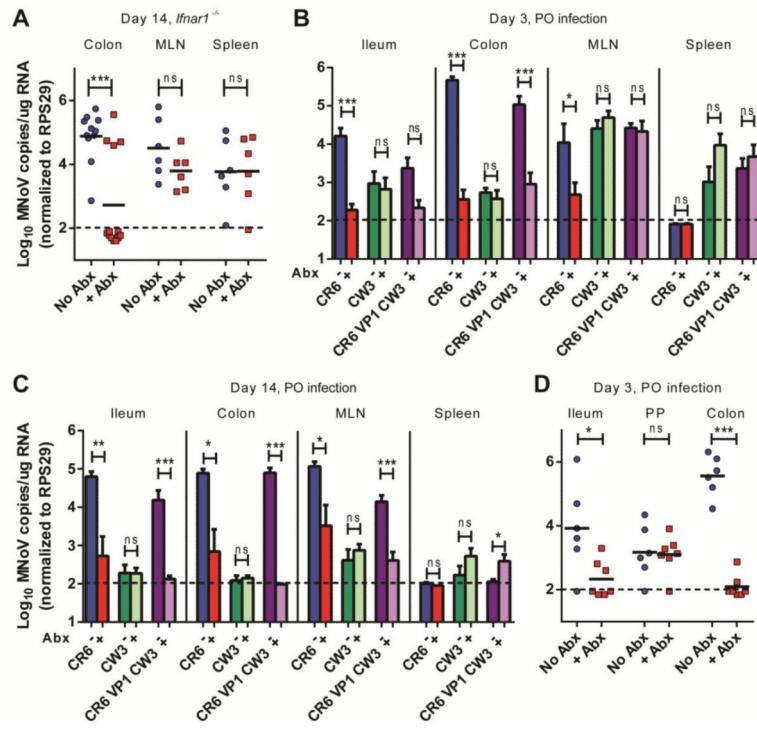


Fig. 3. The intestinal microbiota is dispensable for extraintestinal infection of MNoV and initial viral trafficking

(A) MNoV genome copies in indicated tissues at day 14 after oral CR6 infection of *Ifnar1*^{-/-} mice. Analyzed by Mann-Whitney test; $N = 6$ to 14 mice per cohort combined from three independent experiments. (B and C) MNoV genome copies detected in tissues at day 3 (B) or day 14 (C) after oral CR6, CW3, or CR6^{VP1-CW3} infection. Results from each tissue type were analyzed by one-way ANOVA with Tukey's multiple-comparisons test. ANOVA $P < 0.0001$ except day 14 spleen, where ANOVA $P < 0.01$; $N = 6$ to 11 mice per cohort combined from three independent experiments. (D) MNoV genome copies detected in the indicated tissues (PP, Peyer's patches; Ileum, ileal tissue with no PP) at day 3 after oral CR6 infection. Analyzed by Mann-Whitney test; $N = 6$ or 7 mice per cohort combined from three independent experiments. * $P < 0.05$, *** $P < 0.001$; ns, not significant.

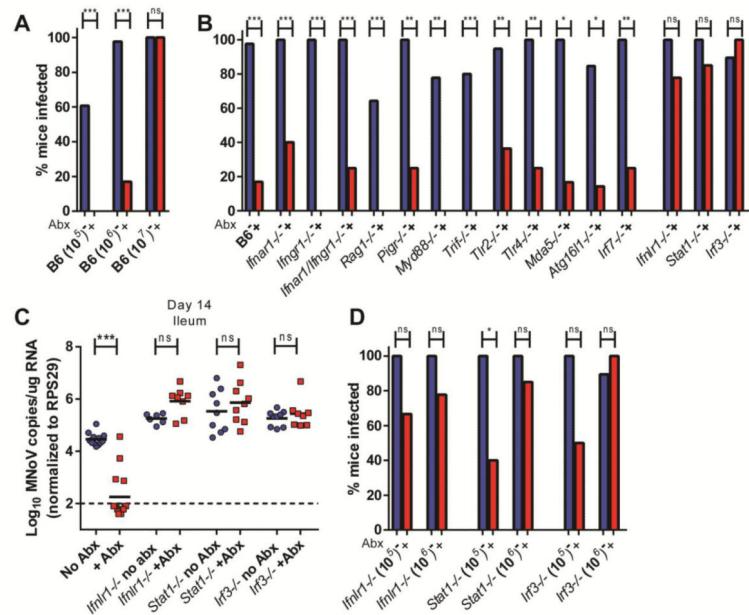


Fig. 4. The IFN-λ pathway regulates antibiotic-dependent clearance of CR6

(A) MNoV was detected in fecal pellets of control B6 mice at day 7 after infection of 10⁵, 10⁶, or 10⁷ pfu of CR6. Percentages of infected mice are shown. Numbers of mice are shown in table S1. Analyzed by contingency table analysis with Fisher's exact test. (B) Mice deficient for the indicated genes or control mice (B6) were pretreated with Abx, then infected with 10⁶ pfu of CR6. Infectivity was assessed according to detection of CR6 in fecal pellets at day 7 after infection; percentages of mice that became infected are shown. Numbers of mice are shown in table S1. Analyzed by contingency table analysis with Fisher's exact test. Each genotype was assessed in at least three independent experiments. (C) MNoV genome copies detected in ileum at day 14 after CR6 infection. Analyzed by one-way ANOVA with Tukey's multiple-comparisons test. ANOVA $P < 0.0001$; $N = 6$ to 15 mice per cohort combined from two to seven independent experiments. (D) MNoV was detected in fecal pellets at day 7 after infection with 10⁵ or 10⁶ pfu of CR6. Percentages of infected mice are shown. Numbers of mice are shown in table S1. Analyzed by contingency table analysis with Fisher's exact test. Each genotype was assessed in at least four independent experiments. Comparisons of +Abx, 10⁵ pfu of CR6 infections to B6, +Abx, 10⁵ pfu of CR6 infections shown in (A) by Fisher's exact test are all $P < 0.01$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.