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# Effects Of Mouse Hepatitis Virus Infection On The Resistance Of Mice To Salmonella Typhimurium.

Michael Todd Fallon
*University of Alabama at Birmingham*

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# Effects of mouse hepatitis virus infection on the resistance of mice to *Salmonella typhimurium*

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# EFFECTS OF MOUSE HEPATITIS VIRUS INFECTION ON THE RESISTANCE OF MICE TO SALMONELLA TYPHIMURIUM

by

MICHAEL TODD FALLON

A DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Pathology in
the Department of Comparative Medicine, in
the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

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Name of Candidate: Michael Todd Fallon

Title: Effects of Mouse Hepatitis Virus Infection on the Resistance of Mice to Salmonella typhimurium

The mechanisms by which mouse hepatitis virus (MHV) field strain UAB modulates resistance to Salmonella typhimurium infections in mice were studied. Resistance to salmonella in mice was monitored by quantitative bacterial colony counts of the spleen and liver. A general observation was that intranasal MHV infection of euthymic mice increased host resistance to intravenous salmonella infection when the interval between MHV and salmonella infections was 6 to 8 days, but decreased resistance when the interval was 1 to 3 days. Much higher doses of salmonella were necessary to observe increased resistance to salmonella infection 6 days after MHV infection in $Ity^r$ mice than in $Ity^s$ mice. The Lps and Xid loci did not alter the effect of MHV infection on salmonella resistance. However, in contrast to these effects in euthymic mice, MHV infection of athymic nude mice was markedly detrimental to salmonella resistance, even at low MHV doses. This suggested that T cells may play a role in the modulation of salmonella resistance by MHV.

To determine whether or not the MHV-induced resistance was due to suppression of salmonella growth or to enhanced killing, studies were performed

with an Aro$^-$ salmonella strain that could not grow in vivo and a salmonella strain bearing the pHSG422 temperature-sensitive plasmid to monitor both salmonella growth and killing. The results suggested that MHV infection enhances suppression of salmonella growth, but also may inhibit killing during the early clearance phase following salmonella injection.

Because large amounts of type I interferon (IFN) were detected in the spleens and serum of mice infected with MHV-UAB, the role of type I IFN in the MHV-induced increase in salmonella resistance was investigated. The effect of the potent type I IFN inducer poly I:C was qualitatively similar to that of MHV infection on salmonella resistance, and neutralizing antiserum directed against type I IFN significantly inhibited the induction of resistance to salmonella by MHV infection. Thus, it appears that the modulation of salmonella resistance by MHV infection is at least partially mediated by type I IFN.

Abstract Approved by: Committee Chairman Trenton R. Schoeb

Program Director Samford P. Bishop

Date:_____ Dean of Graduate School Jerry L. Hickey

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I also thank Dr. Bill Benjamin, for sharing his time and expertise in mouse and salmonella genetics, and Dr. Doug Weigent, for sharing his time and expertise in the interferon studies. Without these two individuals much of the work would have been impossible. Special thanks go to my colleague, Dr. Mary Reinhard, for her discussions and infectious enthusiasm about immune mediators, and to Dr. Sue Michalek, whose in vitro expertise enabled me to avoid some potential problems that emerged during the course of the work.

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Last, but certainly not least, I wish to express my heartfelt gratitude to my wife, Deborah Talkington, for her suggestions concerning this work and for her unending love and support which carried me through many tough times.

## DEDICATION

**This manuscript is dedicated to the memory of my grandfather,**

**Russel W. Trumbore**

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# INTRODUCTION

The manuscripts that follow describe studies of modulation of resistance to Salmonella typhimurium in mice by infection with a strain of mouse hepatitis virus (MHV), a murine coronavirus. The original impetus for this work was an observation that MHV infection was present in mice affected by a natural salmonella epizootic; because MHV infections are known to alter many immunologic responses, it seemed reasonable that MHV infection could have had a role in the expression of the observed disease. However, studies of the interactions of salmonella and MHV infections are applicable to other questions of broader significance, including pathogenesis and host effects of MHV, mechanisms of resistance to salmonella infection, mechanisms of interactions between viral infections and secondary bacterial disease, and the possibility of therapeutic use of viruses or virus components to stimulate defensive responses against intracellular pathogens and perhaps even neoplasms.

The term MHV actually designates a group of related coronaviruses; however, MHV strains having similar antigenic characteristics and RNA electrophoretic patterns can have widely differing tissue tropisms and pathogenic properties (31). MHV is shed in feces and aerosols of respiratory secretions (1,49,50) and can be transmitted among mice by transfer of transplantable tumors

(14,20). It is very infectious, so prevalence of MHV infection in conventionally maintained mouse colonies can approach 100% (35,44).

Expression of MHV disease is dependent upon many factors, including virus strain (4,61), mouse strain (37,59), age of mice (47,58), immune status of mice (18,64), concurrent disease (32,63,68), and experimental manipulations (40,48,69). Subclinical enzootic infections are the rule (1,28,36), but epizootic infections with high morbidity and mortality can occur in susceptible, non-immune mice (1,3). In euthymic mice, MHV infections follow two overlapping general patterns which vary depending on the various tissue tropisms of the MHV isolates (2). In the respiratory pattern, which is the most common, the nasal passages and lungs are infected initially, with dissemination to lymph nodes, thymus, spleen, brain, liver, and intestine via the bloodstream. In the enteric pattern, infection is more restricted to the nasal passages and bowel, with variable spread to other organs, such as liver and abdominal lymph nodes. A few strains are neurotropic, causing demyelinating encephalitis. In athymic (nude) mice, infections with virulent MHV strains are characterized by acute fatal hepatitis, but less virulent strains often cause chronic hepatitis resulting in progressive weight loss and, eventually, death (21,62).

Immunity to MHV is incompletely understood. Serum antibody has not been found consistently to be protective (28), but cell-mediated immunity clearly is important because nude and neonatally thymectomized mice are very susceptible to MHV (55). The roles of natural killer (NK) cells and interferons (IFNs) are unclear (46,52,53,57), although depletion of NK cells can result in increased MHV replication in the liver and spleen (11), and some reports indicate a positive effect of IFNs on resistance to MHV infections (22,47,60,65,66,67). However, results of

other studies suggest that IFN may not protect mice from MHV infections (52). Furthermore, MHV infections of some mouse strains (46) and cell cultures (23) do not appear to induce large amounts of IFN.

MHV infection has a variety of effects on the host that can complicate the experimental use of mice. For example, MHV infection can induce T-lymphocyte differentiation antigens on spleen and lymph node lymphocytes in nude mice (51), and thymocyte cultures infected with MHV have reduced mitogenic responses to concanavalin A (30). Inapparent MHV infection can alter ectoenzyme phenotypes of bone marrow and peritoneal macrophages (16) and inhibit antibody responses to both T-dependent and T-independent antigens (34). In contrast, MHV infection of mice can enhance their ability to mount a humoral response to sheep erythrocytes (67). MHV also can interact with other murine pathogens, including Eperythrozoan coccoides (32), K virus (63), retroviruses (38), Schistosoma mansoni (68), encephalomyocarditis virus (12), and pneumonia virus of mice and Sendai virus (16).

Salmonella typhimurium infections in mice have been studied extensively as a model of human typhoid fever (41). In an initial clearance phase, nearly all intravenously injected salmonella are cleared from the blood, and over 90% of the cleared salmonella are killed during the first two hours after injection (5). Of the survivors, over 90% are found in the liver and spleen (5,41). Salmonella that survive the initial clearance phase do not appear to differ genetically from those killed, but seem to be protected from further killing in an unidentified site where they are able to replicate (6,41). The actual physical location of the protected site has not been ascertained, but probably is intracellular (8). Salmonella readily enter macrophages (41), but it is not known if salmonella chronically reside there.

Growth of salmonella in the protected site is affected by both salmonella gene loci, such as mviA (7), and mouse gene loci, such as Ity (9), but the mechanisms for these effects are not clear. During the later stages of experimental disease, extracellular replication occurs and may predominate (24).

In mice of resistant genotypes, the onset of specific immune responses is associated with a decline in the rate of salmonella growth and a net decrease in numbers of salmonella in the host's tissues (5,41), but it is not known how these effects occur. Antibody-mediated immunity to salmonella has been difficult to demonstrate (19), and most S. typhimurium strains are resistant to complement-mediated lysis, even in the presence of specific antibody (10). Macrophages probably are responsible for most killing of salmonella because mice treated with silica particles to block phagocyte function are more susceptible to salmonella infection (42), as are $Lps^d$ mice whose macrophages are not stimulated by bacterial lipopolysaccharide (LPS) (41). Nude mice do not appear to be more susceptible than euthymic mice until about 10-14 days after salmonella challenge (43), and attempts to transfer resistance with T-lymphocytes usually have failed (13,26,29); therefore, immunity to salmonella appears to be largely dependent on non-specific defenses during the first two weeks after experimental inoculation, with specific immunity having a role at later times under some circumstances.

Studies of the effects of MHV infection on resistance to experimental salmonellosis may also be helpful in elucidating possible mechanisms of interactions between other viral and bacterial infections. Such interactions have been recognized as major problems in human and veterinary medicine for nearly a century. For example, in past human influenza epidemics, many deaths resulted

from systemic or respiratory disease caused by secondary Streptococcus pneumoniae, Hemophilus influenzae, Escherichia coli, Staphylococcus aureus, and Neisseria meningitidis infections (39), and the incidence of otitis media and recurrent otitis media caused by S. pneumoniae and H. influenzae has been found to be significantly higher in children with influenza A or B virus, respiratory syncytial virus, or adenovirus infection (25). Swine influenza also commonly is complicated by pneumonia caused by H. influenzae (56), and in cattle and sheep, severe pneumonia caused by Pasteurella hemolytica commonly follows respiratory viral infections (15,33). Although much has been learned through experimental studies, the precise mechanisms of viral-bacterial interactions still are not clearly understood (39).

The studies comprising this work were done in three phases in which successive questions were addressed. I began by testing whether or not MHV infection can affect resistance to salmonellosis and found that immunocompetent mice infected with MHV were transiently more, rather than less, resistant than control mice to salmonellosis, as determined by survival curves and numbers of salmonella in livers and spleens. This effect was more pronounced in salmonella-susceptible $Ity^s$ mice than in resistant $Ity^r$ mice. Because the Ity locus primarily affects the ability of the host to limit the number of intracellular salmonella, the results suggest that MHV restores this ability in $Ity^s$ mice. Mice having the $Lps^d$ mutation, whose macrophages are unresponsive to bacterial lipopolysaccharide and thus have reduced ability to kill or inhibit growth of salmonella, and mice having the Xid mutation, which have deficient antibody responses to T-dependent antigens, did not have altered resistance to salmonella after MHV infection. In contrast to

euthymic mice, athymic nude mice were rendered more, rather than less, susceptible to salmonella by MHV infection. Because nude mice not infected with MHV are no more susceptible to salmonella than euthymic mice (sacrifice 4 days after salmonella infection), these results suggest that T-lymphocytes can contribute to resistance to salmonella. In euthymic mice resistance to salmonella infection may be mediated by stimulation of effector cell(s) by T cells, whereas in nude mice non-T cell dependent resistance mechanisms compensate for the lack of T cells. In the presence of MHV infection, the non-T cell-dependent compensatory salmonella resistance mechanisms may be overwhelmed, resulting in decreased rather than increased resistance to salmonella infection following MHV infection.

The second series of experiments addressed the question of whether MHV infection enhances resistance to salmonella by stimulating the host's ability to inhibit salmonella growth or by stimulating killing of salmonella. MHV infection did not reduce the numbers of Aro$^-$ mutant salmonella, which are incapable of in vivo growth, in the liver or spleen, nor did it affect numbers of salmonella bearing a temperature-sensitive plasmid that could not replicate at host body temperature. These results indicate that MHV infection induced resistance to salmonella infection primarily through effects on growth suppression.

Because MHV infection is reported to stimulate interferon (IFN) release and there is evidence that IFN can stimulate resistance to salmonella, the relationship between induction of IFN and stimulation of resistance to salmonella by MHV infection was examined. Large amounts of type I IFN were induced by MHV infection, and the type I IFN inducer, polyinosinic:polycytidilic acid (poly I:C), was able to qualitatively mimic the effects of MHV infection on salmonella resistance,

although effects of lesser magnitude were produced. The effects of both MHV infection and poly I:C treatment were significantly reduced by neutralizing antibody specific for type I IFN, indicating that type I IFN has a significant role in the enhancement of resistance to salmonella induced by MHV infection. Paradoxically, the neutralizing antibody increased the resistance of mice given only salmonella. This suggests that IFN release by the host in response to salmonella infection may not be beneficial to host resistance against salmonella infection.

MANUSCRIPT #1

# MODULATION OF RESISTANCE TO SALMONELLA TYPHIMURIUM INFECTION IN MICE BY MOUSE HEPATITIS VIRUS (MHV)

By

Michael T. Fallon, Trenton R. Schoeb, William H. Benjamin Jr.,

J. Russell Lindsey, and David E. Briles

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## ABSTRACT

Prior infection of mice with a field strain of mouse hepatitis virus (MHV) increased the early resistance of euthymic mice to virulent Salmonella typhimurium strain SR-11 infections (as defined by significantly fewer salmonella colony-forming units (CFU) present in spleens and livers 4 days after salmonella infection). This increase in salmonella resistance was observed when the interval between MHV and salmonella infections was 6 days, but not at 3, 10, or 14 day intervals. The mouse Ity locus, which controls the number of intracellular salmonella, had a significant effect on the ability of MHV to induce resistance to salmonella. MHV caused an increase in resistance to salmonella in $Ity^s$ (salmonella susceptible) mice at all doses of salmonella tested (100 to 10,000 CFU). In the $Ity^r$ (salmonella resistant) mice tested the beneficial effect of MHV on salmonella resistance was small and when observed, was only present at salmonella doses of 10,000 CFU or greater. Neither the $Lps^d$ nor Xid mutations affected the ability of MHV to increase resistance to salmonella infection. In contrast to euthymic mice, MHV infection greatly decreased the resistance of athymic (nude) mice to salmonella infection. Since the Nu locus does not effect the resistance of mice to salmonella (at 4 days post salmonella infection), these results indicate that MHV infection and the nude phenotype interact to increase susceptibility to salmonella.

These findings reemphasize the importance of keeping laboratory mice used in research free of MHV and other immunomodulatory pathogens.

# INTRODUCTION

It is well documented that viral infections can alter host resistance to secondary bacterial infections, the best known being viral infections that potentiate secondary bacterial infections (1,2). For example, human influenza patients are at an increased risk of either systemic or pulmonary infections caused by Streptococcus pneumoniae, Staphylococcus aureus, Hemophilus influenzae, Escherichia coli, and Neisseria meningitidis (3,4). However, it has been demonstrated that experimental influenza virus infections of mice can increase or decrease resistance to Listeria monocytogenes, depending partly on the interval between the infections (5). The mechanisms by which viruses alter host resistance to bacterial pathogens are poorly understood (1).

We investigated the ability of a field strain of mouse hepatitis virus (MHV) to alter the early resistance of inbred mice to Salmonella typhimurium infections. We found that MHV infection of euthymic mice resulted in increased resistance (as defined by decreased numbers of salmonella in spleens and livers) to experimental salmonella infections when there was a 6 day interval between MHV and salmonella infections. We also found that the Ity locus affects the ability of MHV to increase resistance of euthymic mice to salmonella. A very different result was obtained with athymic (nude) mice, which had decreased resistance to salmonella following MHV infection. These findings suggest that the ability of MHV to increase resistance to salmonella may be related to the amount of intracellular salmonella growth, and that functional T cells are necessary for MHV to enhance resistance to salmonella infection.

## RESULTS

### MHV infection can increase the resistance of A/JCr and BALB/cAnNCr mice to salmonella

In initial experiments, we studied the effect of varying the interval between MHV and salmonella infections in A/JCr and BALB/cAnNCr mice, which are resistant and susceptible, respectively, to both MHV and salmonella infections. MHV-infected groups of A/JCr and BALB/cAnNCr mice received 100 plaque-forming units (PFU) of MHV intranasally, then 10,000 salmonella colony-forming units (CFU) intravenously 3, 6, 10, or 14 days later. All mice were sacrificed 4 days after receiving salmonella. When compared with control mice that received salmonella but no MHV, MHV-infected A/JCr and BALB/cAnNCr mice had significantly fewer salmonella in spleens and livers at a 6 day MHV-salmonella interval (largest effect in the BALB/cAnNCr mice), and MHV-infected A/JCr mice had significantly more salmonella in spleens and livers at a 3 day interval (Figure 1). Significant differences were not found at other intervals. This finding in A/JCr mice that a viral pathogen could decrease or increase resistance to a bacterial pathogen, depending on the interval between the viral and bacterial infections, has been reported previously in mice given influenza virus and Listeria monocytogenes (5). To determine whether or not the interval between salmonella infection and mouse sacrifice was important, groups of A/JCr and BALB/cAnNCr mice received 100 MHV PFU, then 10,000 salmonella CFU 6 days later. In comparison with mice that received only salmonella, MHV-infected mice of both mouse strains had significantly fewer salmonella in spleens and livers 3, 4, and 5 days after salmonella infection (Figure 2).

The ability of MHV to increase resistance to salmonella is dose dependent in A/J Ity$^r$ mice, but not in BALB/cAnNCr Ity$^s$ mice

To determine how the salmonella dose affected the ability of MHV to enhance resistance to salmonella, A/J mice were infected with 100 PFU of MHV, then with 5,000, 10,000 or 50,000 salmonella CFU 6 days later. At sacrifice 4 days after salmonella infection, the number of salmonella in spleens and livers was compared with matched control groups that received only salmonella. There was no difference between MHV-infected and control mice receiving 5,000 or 10,000 CFU, but at the 50,000 CFU dose, MHV-infected mice had significantly fewer salmonella than did control mice (Figure 3).

BALB/cAnNCr mice were used in a similar experiment. Groups received 100 MHV PFU, then 100, 1,000, 5,000, or 10,000 salmonella CFU 6 days later. MHV-infected mice in all salmonella dose groups had significantly fewer salmonella than did control mice not receiving MHV (Figure 3).

The effect of MHV on salmonella resistance in other inbred mouse strains

To gain information about specific mechanisms responsible for mediating the MHV-induced increase in early resistance of mice to salmonella infection, inbred mouse strains were studied with different Ity alleles or other known genetic defects influencing host immune function.

Ity. If other background genes are held constant, Ity$^r$ mice are more resistant to salmonella infection than Ity$^s$ mice. Although there has been some controversy about the mechanism of action of this locus (6-8), recent data indicate that Ity$^r$ mice are better able to control the growth rate of intracellular salmonella (7, and W.H. Benjamin, Jr. and D.E. Briles, manuscript in preparation). BALB/cPt

($\underline{Ity}^{s/s}$) and C.D2 Idh-$1^b$-$Pep3^b$ ($\underline{Ity}^{r/r}$) mice, congenic for a piece of chromosome 1 containing the Ity locus (9), were given 100 PFU of MHV, then 10,000 salmonella CFU 6 days later. When sacrificed 4 days after receiving salmonella, MHV-infected $\underline{Ity}^{s/s}$ mice had significantly fewer salmonella in both livers and spleens than did control mice given salmonella but not MHV. In $\underline{Ity}^{r/r}$ mice, no significant difference was found between mice given MHV and salmonella, and mice given only salmonella (Figure 4).

Lps. C3H/HeJCr-$\underline{Lps}^{d/d}$ mice, whose macrophages are incapable of effectively killing intracellular salmonella (10), and normal C3H/HeNCr-$\underline{Lps}^{n/n}$ mice were given 100 MHV PFU, then 10,000 salmonella CFU 6 days later. There was no significant difference between MHV-infected mice and control mice (salmonella only) in the number of salmonella in spleens and livers at sacrifice 4 days after salmonella infection. However, at a salmonella dose of 30,000 CFU, MHV-infected mice of both strains had significantly fewer salmonella at sacrifice in both spleens and livers than did control mice that received salmonella only (Figure 5).

Xid. Experiments as described above for C3H/HeJCr and C3H/HeNCr mice were performed with CBA/NCr-xid/xid mice having B cell defects in T-independent antibody production (11), and normal CBA/JCr mice. As in the C3H mice, at a dose of 10,000 salmonella CFU, there was no difference in the number of salmonella in spleens and livers between MHV-infected and control mice, but at a dose of 30,000 CFU, MHV-infected mice had significantly fewer salmonella than did control mice of both mouse strains (data not shown). In concert with results from the A/J and C3H (/HejCr and /HeNCr) strains in which MHV-infected mice that received salmonella doses of 50,000 CFU (Figure 3) and 30,000 CFU (Figure

5) respectively had fewer salmonella than controls that received no MHV while MHV-infected mice receiving 10,000 CFU did not, these results suggested that for MHV to reduce salmonella recovery, a certain minimum dose of salmonella must be given.

Nu. To study the effect of competent T cell function on the ability of MHV to modulate resistance to salmonella, congenic BALB/cAnNCr-nu/+ (heterozygote) and -nu/nu (nude) mice were given 100 PFU of MHV, then 10,000 salmonella CFU 6 days later. As expected, heterozygotes with normal T cell function given 100 PFU of MHV had significantly fewer salmonella in spleens and livers than did control heterozygotes that received salmonella only. In contrast, only 1 of 5 nude mice given MHV survived until sacrifice 4 days after salmonella infection (data not shown). The experiment was repeated, but instead of sacrificing the mice after salmonella infection, mice were observed until dead. The survival curves (Figure 6) illustrate that MHV increased the resistance of heterozygote mice to salmonella, but decreased the resistance of nude mice.

To determine what effect the MHV dose had on salmonella resistance in heterozygote and nude mice, 12-week-old nude and heterozygote mice were given tenfold dilutions of MHV from 10 to $10^{-4}$ PFU, then 10,000 salmonella CFU 6 days later. Control groups received salmonella but no MHV. In the heterozygotes, decreasing MHV doses resulted in a loss of the MHV-induced increase in salmonella resistance, but in the nude mice some mice died in all dose groups and survivors had significantly more salmonella than did control nudes that received salmonella only (Figure 7). Thus, the absence of functional T cells in the nude mice prevented the enhancement of salmonella resistance by MHV infection.

To verify that the mortality experienced by nude mice given both MHV and salmonella was not due to MHV alone, similar experiments were performed in 18- and 22-week-old nude mice, with further decreases in virus dose and the addition of groups that received MHV but no salmonella to control for mortality caused by MHV. No dose of MHV was found that enhanced the resistance of nude mice to salmonella. In the 18-week-old nu/nu mice, a dose of at least $10^{-2}$ PFU was required to observe a detrimental effect of MHV on salmonella resistance, whereas in the 22-week-old mice a dose of at least 1 PFU was necessary. Eighteen-week-old mice that received 1 PFU or $10^{-2}$ PFU of MHV and salmonella had higher mortality than mice that received the same doses of MHV alone, and 22-week-old nu/nu mice that received $10^{-2}$ PFU of MHV and salmonella had higher mortality than mice given MHV alone. It appears that MHV alone was not responsible for the mortality seen in nudes receiving both MHV and salmonella, although at 1 of the 4 MHV doses that resulted in some mortality (the 1 MHV PFU dose in 22-week-old mice), mice that received MHV and salmonella had the same mortality as mice that received MHV only (Figure 8).

## DISCUSSION

Our most striking observation was that at the 6 day MHV-salmonella infection interval, MHV infections resulted in an increase in resistance to Salmonella typhimurium in BALB/cAnNCr-nu/+ mice, but a decrease in resistance in BALB/cAnNCr-nu/nu mice. An increase in salmonella resistance similar to that seen in the BALB/cAnNCr euthymic mice was also seen in BALB/cPt mice. Both of these BALB/c sublines have the $Ity^s$ genotype, which makes them highly susceptible to salmonella infection by allowing rapid intracellular growth of S.

typhimurium. When congenic Ity$^r$ mice were infected with the same regimen of MHV and S. typhimurium (100 PFU MHV, then 10,000 salmonella CFU 6 days later), MHV did not significantly change their resistance to salmonella. Thus, it appears that for MHV to cause a change in resistance to salmonella, rapid salmonella growth must be present, suggesting that MHV may reduce the salmonella growth rate in Ity$^s$ mice.

The inability of MHV to alter resistance to S. typhimurium in congenic Ity$^r$ mice was consistent with a lack of an MHV-induced increase in resistance at a dose of 10,000 S. typhimurium CFU in the Ity$^r$ CBA, C3H, and A/J (Jackson Laboratories) strain. However, in all of the latter Ity$^r$ strains, it was possible to see an MHV-mediated increase in resistance at higher salmonella doses. These results, and the data from the Ity$^s$ mice, suggest an alternate hypothesis that the ability of MHV to alter resistance to salmonella in euthymic mice depends on the stage of inflammation or amount of inflammation present in internal organs (i.e. the more salmonella, the more inflammation), and that MHV could act by increasing the rate of salmonella killing. Distinguishing between these competing hypotheses (growth inhibition versus increased killing) will have to be addressed by further experimentation. We observed that at a 6 day MHV-salmonella interval A/JCr mice given 100 PFU MHV and 10,000 salmonella CFU were significantly more resistant to salmonella than mice given only salmonella, while A/J (Jackson) mice given MHV and salmonella under identical conditions were not. This is probably due to unknown differences in the genotypes of the 2 sublines.

Several hypotheses must be considered to explain the fact that the nu/nu genotype does not alter early resistance to salmonella in non-MHV-infected mice,

but adversely affects resistance to salmonella infection in MHV-infected mice. Previous observations by others that MHV infections can result in macrophage activation (11,12) could account for the increase in resistance to S. typhimurium we found in euthymic mice. Because nude mice and thymectomized mice are more susceptible to MHV infection (13), it appears that T cells or their lymphokines are necessary for macrophages to successfully control MHV infections, and perhaps become activated to resist salmonella infections. Accordingly, in the absence of functional T cells, macrophages in nude mice may be killed or functionally impaired by MHV infection, resulting in decreased resistance to salmonella infection.

It is known that nude mice compensate in part for their lack of functional T cells with increased macrophage (14) and natural killer cell function (15) under normal circumstances. Thus, another possibility is that T cells contribute to the non-specific early resistance of normal mice to salmonella, and that in nude mice, anti-salmonella activities of macrophages and NK cells successfully compensate for the absence of functional T cells. Following this line of reasoning, MHV may stimulate T cell dependent immune functions in euthymic mice while adversely affecting non-T cell compensatory mechanisms of resistance to salmonella in nude mice. However, attempts by others to transfer salmonella resistance with immune T cells have generally failed (16,17).

It is well established that there are genetic differences between euthymic mouse strains that can influence their susceptibility to MHV infections (18-20). The exact hierarchy of mouse strain susceptibility to MHV depends on the MHV strain, but usually A/J mice are resistant, C57BL/6 (and sometimes BALB/c) mice are susceptible, and other mouse strains have intermediate susceptibilities. We

have not yet quantitated the amount of MHV-UAB present in tissues of different strains of mice, and thus we do not know if MHV-UAB grows significantly better in some euthymic mouse strains than others. However, our Ity studies included the use of Ity congenic mice, and we feel that it is very unlikely that our results are due to differences in the ability of euthymic mouse strains to support viral replication.

Although most reports of MHV strains altering host immune function have focused on adverse effects (21), there have been several reports of MHV strains increasing the resistance of mice to other viral pathogens. MHV infections have increased host resistance to Sendai virus and pneumonia virus of mice (22), and also to encephalomyocarditis virus (23).

Our studies have shown that MHV can significantly alter the resistance of mice to salmonella infections, adding to the growing list of immunomodulatory properties of MHV strains in mice. Since MHV infections are widespread, clinically silent, and chronic in breeding populations (21), investigators should be aware of the potential for MHV infections to confound experimental results.

## MATERIALS AND METHODS

Mice. Weanling BALB/cAnNCr, A/JCr, C3H/HeJCr ($Lps^d$), C3H/HeNCr ($Lps^n$), CBA/NCr (Xid), and CBA/JCr mice from the National Cancer Institute breeding facility at Frederick, Maryland, and 10 week old A/J mice from Jackson Laboratories (Bar Harbor, Maine) were received in filter-ported crates. BALB/cPt ($Ity^{s/s}$) and 20th generation C.D2 Idh-$1^b$-$Pep3^b$ ($Ity^{r/r}$) mice (9) congenic for a portion of chromosome 1 known to include the Ity locus (24) were bred from stock graciously provided by Dr. Michael Potter. All mice were maintained in

microisolator cages (Lab Products, Aberdeen, MD) with sterile food, water and bedding. Sera from mice of each strain were screened for antibodies to MHV, Sendai Virus, lymphocytic choriomeningitis virus, mouse rotavirus, pneumonia virus of mice, Reovirus 3, Theiler's GD-VII virus, minute virus of mice, K virus, mouse pox virus, and Mycoplasma pulmonis by Charles Rivers Professional Services (Wilmington, MA) to verify that they were free of these pathogens before experiments began. At the end of about every other experiment, sera from MHV-infected and control mice were screened as above to verify that MHV cross-contamination did not occur between experimental groups and that experiments were not compromised by other pathogens. Unless otherwise noted, mice were used at 9-16 weeks of age. Experimental groups contained 5 or 6 mice, except for the data presented in Figures 1 and 2 in which 3 mice per group were used.

Virus. Frozen aliquots (-70°C) of fourth passage mouse hepatitis virus strain UAB (MHV-UAB) were thawed just prior to use in all experiments. This murine coronavirus is a field strain whose isolation has been previously described (25). Experimental mice were inoculated intranasally (IN) with virus. Titration studies in A/JCr mice indicate that 1 PFU of MHV-UAB is equivalent to 1,000 $Seroconversion_{50}$ units (a 1 unit dose would cause seroconversion to MHV in 50% of mice). Control mice received an equal volume of sterile medium intranasally in place of MHV.

Salmonella. Virulent Salmonella typhimurium strain SR-11 was used in all experiments. Frozen aliquots (-70°C) of bacteria from a single late log phase broth culture were thawed and suspended in sterile saline just prior to injection. Mice were injected with salmonella suspended in 200 ul of sterile saline via tail vein.

Effect of MHV on resistance to salmonella. In experiments mice were divided into experimental groups which were infected first with MHV, then with salmonella, and control groups which were treated identically except sterile medium was substituted for MHV. The number of salmonella CFU in spleens and livers was individually determined. Each organ was homogenized in cold saline with a Stomacher tissue homogenizer (Tekmar Company, Cincinnati, OH), further diluted in cold saline, then plated on Brain-Heart Infusion agar or 5% sheep blood agar (BBL Microbiology Systems, Cockeysville, MD). The number of salmonella recovered from organs was expressed as a base ten logarithm.

Statistical analysis. Data were analyzed by computer (Statistix II software package, NH Analytical Software, Roseville, MN) using the multifactorial analysis of variance (ANOVA) technique. Differences between group geometric means were considered to be significantly different when p was less than 0.05. In the event of significant factor interactions ($p<0.05$), differences between individual means were tested for significance with Scheffe's test (26).

## ACKNOWLEDGEMENTS

This work was supported in part by grants RR00463 and RR07003 from the Animal Resources Branch, Division of Research Resources, National Institutes of Health (NIH), and grant AI15986 from the National Institute for Allergy and Infectious Disease, NIH. The authors thank Charyl Roy and Julie Erwin for their expert technical assistance in these studies, and Dr. Deborah Talkington for critically reviewing the manuscript.

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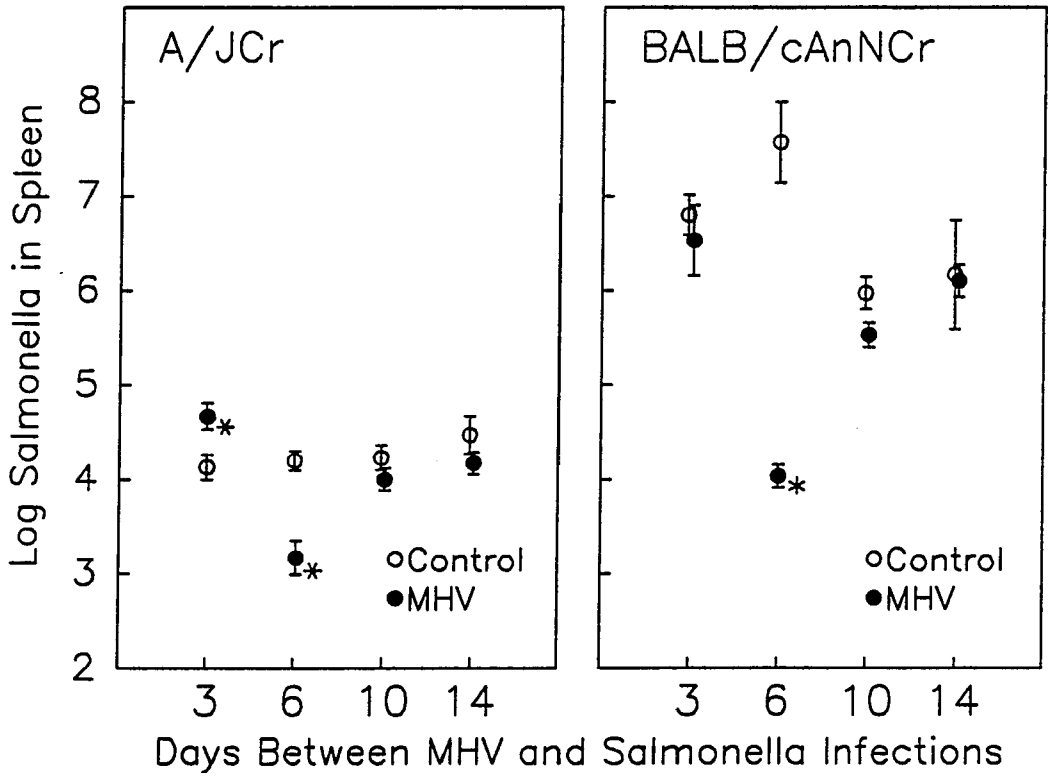


Figure 1. Effect of varying the interval between MHV and salmonella infections in A/JCr and BALB/cAnNCr mice. Experimental mice received 100 PFU of MHV, then 10,000 salmonella CFU 6 days later. Control mice received salmonella only. Points represent geometric means (+/- SE) of salmonella CFU in spleens 4 days after salmonella infection. Asterisks indicate significant differences between experimental and control groups at $p<0.05$.

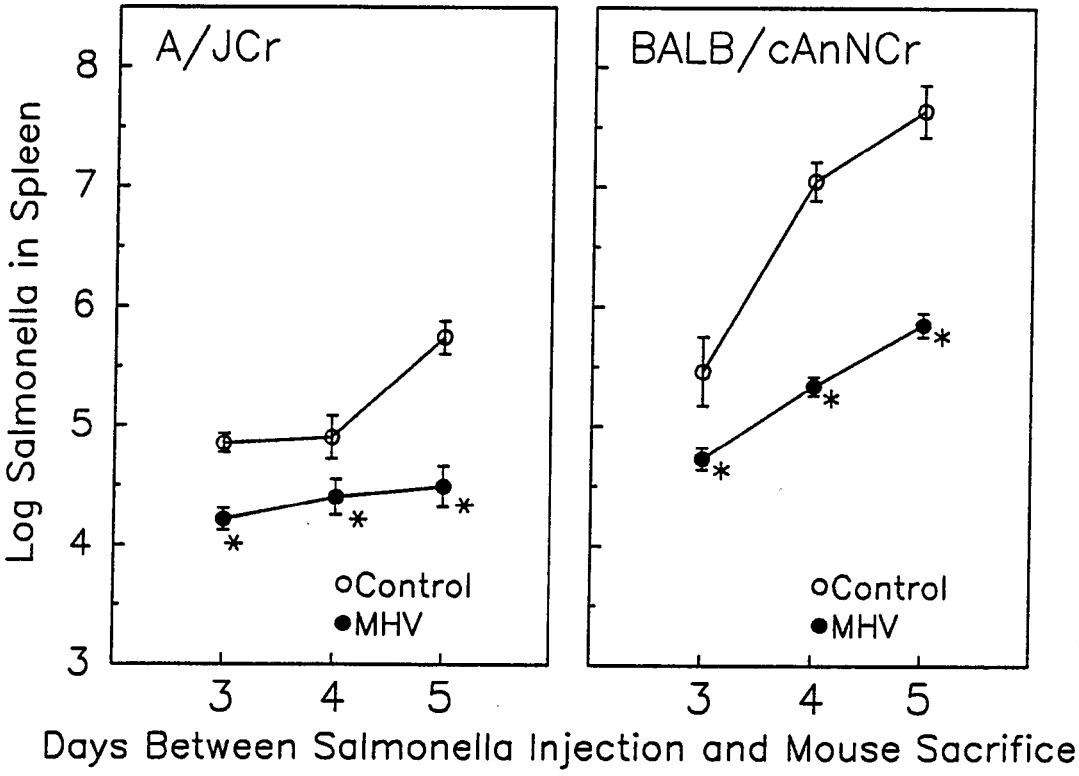


**Figure 2.** Effect of varying the interval between salmonella infection and mouse sacrifice in A/JCr and BALB/cAnNCr mice. Experimental mice received 100 PFU of MHV, then 10,000 salmonella CFU 6 days later. Control mice received salmonella only. Mice were sacrificed at 3, 4, or 5 days after salmonella infection. Points represent geometric means (+/- SE) of salmonella CFU in spleens. Asterisks indicate significant differences between experimental and control groups at $p<0.05$.

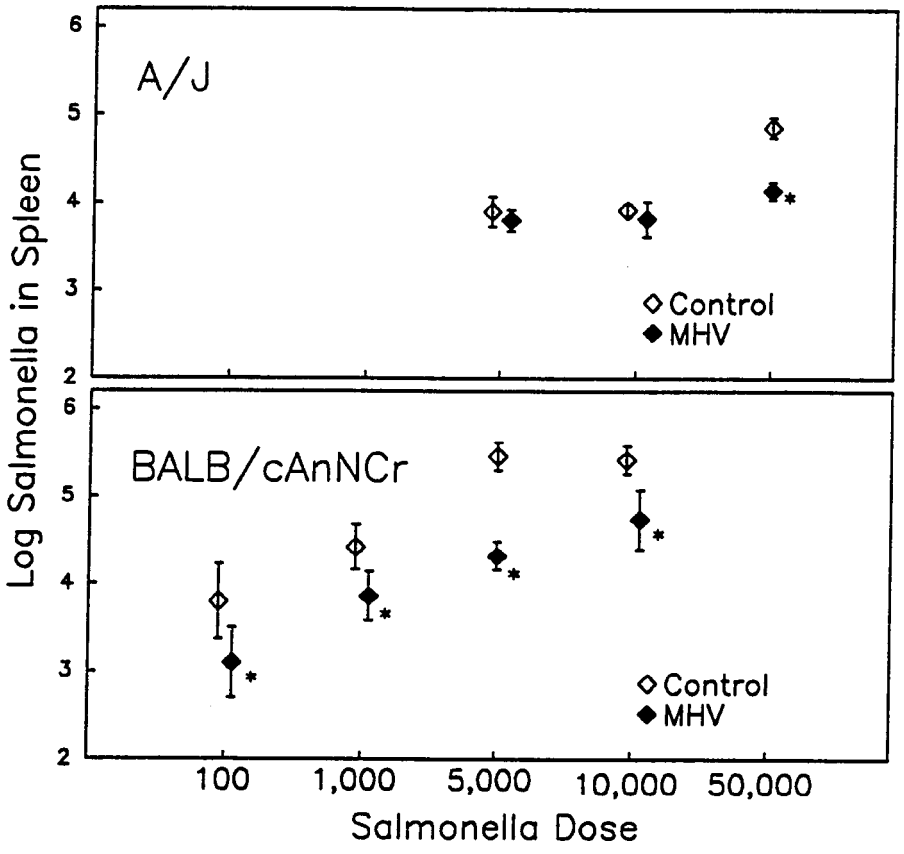


Figure 3. Effect of varying the salmonella dose on the ability of MHV to alter salmonella resistance in A/J and BALB/cAnNCr mice. Experimental mice received 100 PFU of MHV, then varying doses of salmonella 6 days later. Control mice received salmonella only. Points represent geometric means (+/- SE) of the salmonella CFU in spleens 4 days after salmonella infection. Asterisks indicate significant differences between experimental and control groups at $p<0.05$.

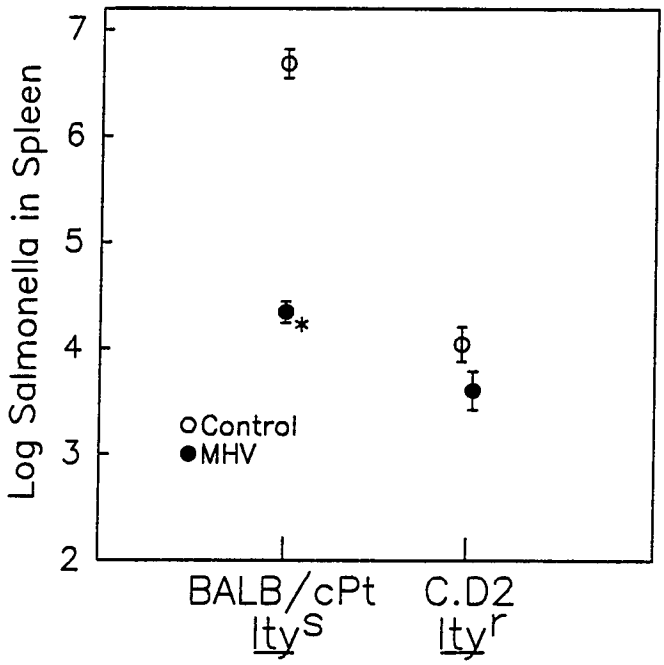


Figure 4. The interaction between MHV and salmonella in mice congenic for Ity. Experimental mice received 100 PFU of MHV, then 10,000 salmonella CFU 6 days later. Control mice received salmonella only. Points represent geometric means (+/- SE) of salmonella CFU in spleens 4 days after salmonella infection. Points represent geometric means (+/- SE) of the number of salmonella in spleens 4 days after salmonella infection. An asterix indicates a significant difference between experimental and control groups at $p<0.05$.

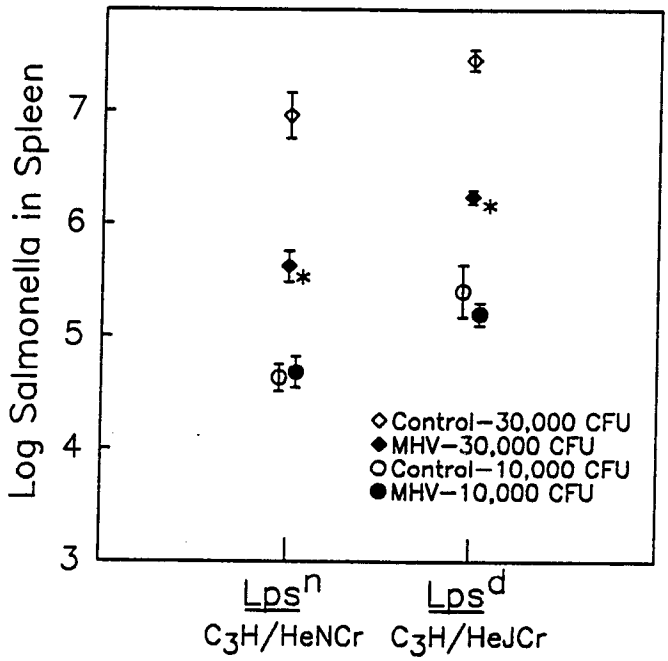


Figure 5. The Lps locus and the MHV-salmonella interaction. Experimental mice (closed symbols) received 100 PFU of MHV, then salmonella 6 days later. Control mice (open symbols) received salmonella only. Mice received either 10,000 CFU (circles) or 30,000 CFU (diamonds). Points represent geometric means (+/- SE) of the number of salmonella in spleens 4 days after salmonella infection. Asterisks indicate significant differences between experimental and control groups at $p<0.05$.

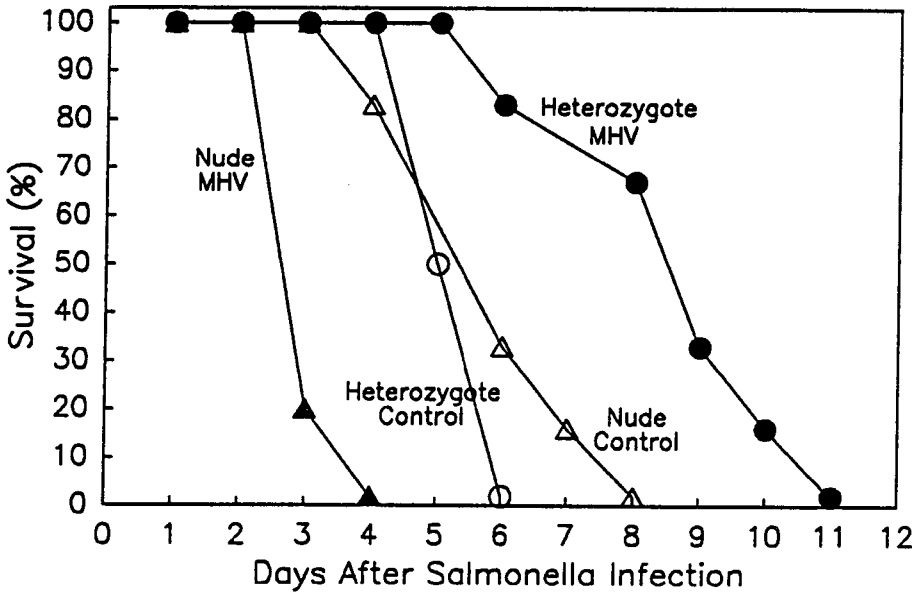


Figure 6. Effect of MHV on the salmonella resistance in nude and heterozygote mice: Survival curves. Experimental mice (closed symbols) received 100 PFU of MHV, then 10,000 salmonella CFU 6 days later. Control mice (open symbols) received salmonella only. BALB/cAnNCr-nu/+ (heterozygote) mice, circles; BALB/cAnNCr-nu/nu (nude) mice, triangles.

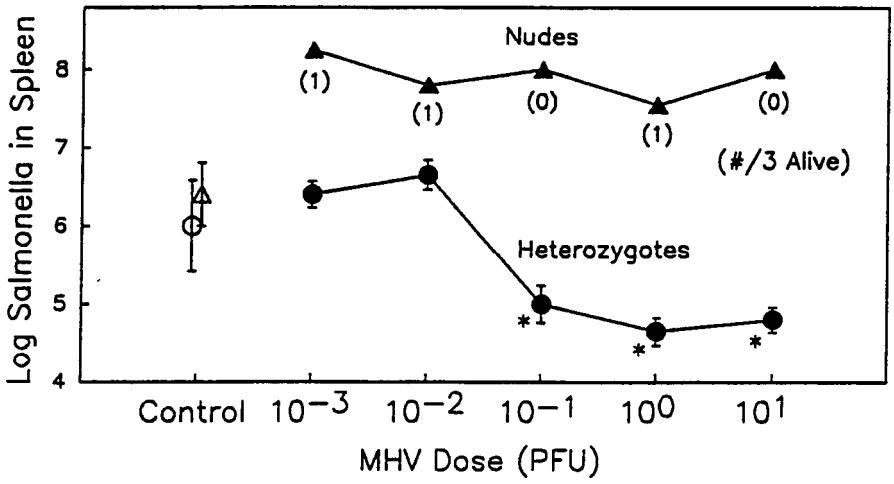


Figure 7. Effect of MHV dose on the salmonella resistance of nude and heterozygote mice: Salmonella recovery. Experimental mice (closed symbols) received varying doses of MHV, then 10,000 salmonella CFU 6 days later. Control mice (open symbols) received salmonella only. BALB/cAnNCr-nu/+ (heterozygote) mice, circles; BALB/cAnNCr-nu/nu (nude) mice, triangles. Points represent geometric means (+/- SE) of salmonella CFU in spleens 4 days after salmonella infection. An asterix indicates a significant difference between a heterozygote experimental group and the heterozygote control group at $p<0.05$. In parentheses below the nude experimental groups are the number of mice that survived to the sacrifice day. Dead mice were not included in the datum except that groups with no survivors were assigned $10^8$ salmonella CFU for graph purposes.

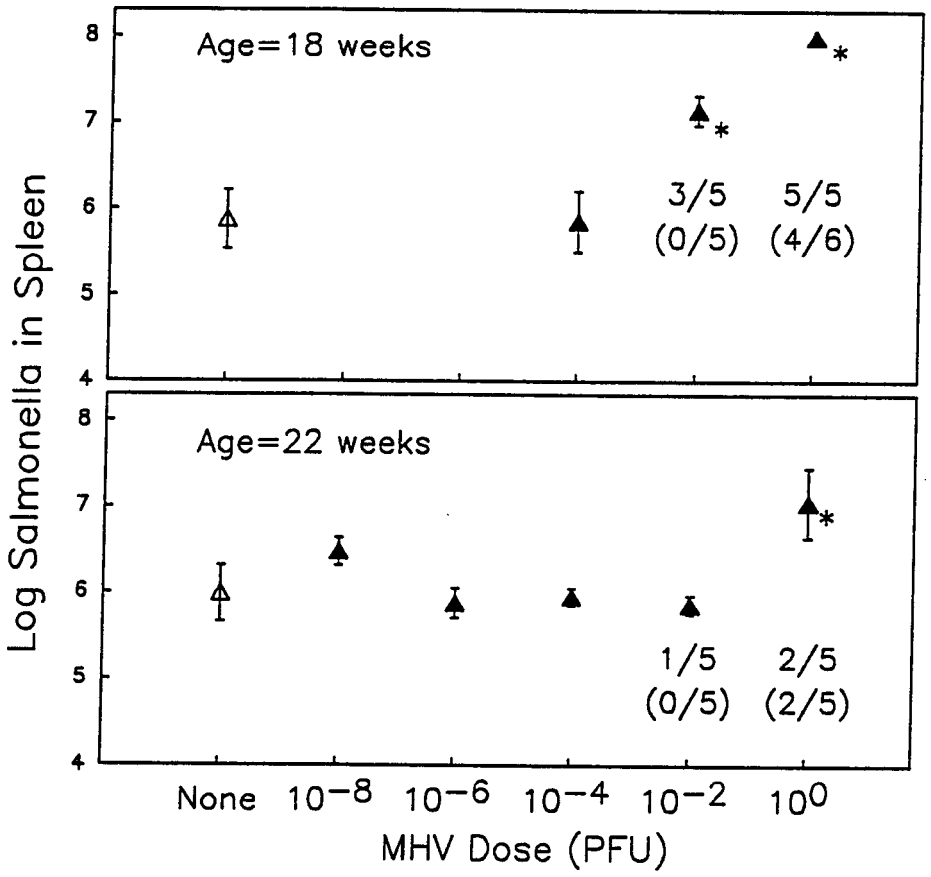


Figure 8. Effect of lower MHV doses on the salmonella resistance of nude mice: Salmonella recovery. Experimental mice received the indicated dose of MHV, then 10,000 salmonella CFU 6 days later. Control mice received salmonella only (MHV dose= "None"). Points represent geometric means (+/- SE) of salmonella CFU in spleens 4 days after salmonella infection. Asterisks indicate significant differences between experimental and control groups at $p<0.05$. Fractions that appear beneath groups represent mortality before the sacrifice day (dead mice/total mice in group). Top fraction, the experimental group (MHV and salmonella); bottom fraction in parentheses, mice that received the indicated MHV dose, but no salmonella. Dead mice were not included in the datum except for the 18 week old 10 PFU group in which dead mice (5 of 5) were assigned $10^8$ spleen salmonella.

Having shown that MHV infection of euthymic mice could enhance resistance to S. typhimurium infection, studies were initiated to determine if the induced resistance was mediated by growth suppression of the salmonella, increased killing of the salmonella, or both. Aside from the salmonella quantitation assays used in the first manuscript, two approaches were used. The first approach involved the use of an Aro$^-$ mutant salmonella strain that could not grow in vivo. Because the salmonella mutants could not grow to replenish bacteria killed by the host, any killing could be observed as a drop in the number of salmonella recovered from the organs. The second approach utilized a salmonella strain bearing a temperature sensitive plasmid that could not replicate at normal mouse body temperature. Thus a drop in the number of salmonella that contained the plasmid in vivo would reflect host killing, and the total number of salmonella recovered from the mice would gauge salmonella growth.

MANUSCRIPT #2

# ENHANCEMENT OF RESISTANCE TO SALMONELLA TYPHIMURIUM IN MICE BY MOUSE HEPATITIS VIRUS INFECTION IS MEDIATED BY HOST SUPPRESSION OF BACTERIAL GROWTH

By

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Prepared for submission to Microbial Pathogenesis

## ABSTRACT

When BALB/cAnNCr mice are infected with mouse hepatitis virus (MHV) then virulent Salmonella typhimurium at a 6 day interval, MHV-infected mice have significantly fewer salmonella four days post salmonella challenge than do controls not infected with MHV. To assess the relative roles of growth suppression and killing of salmonella in the induction of salmonella resistance by MHV infection, we inoculated mice with MHV, then S. typhimurium 6 days later. Four hours after salmonella injection, about 1.5 and 3 times more salmonella were recovered from the spleens and livers, respectively, of MHV-infected mice than from control mice not infected with MHV. This result suggested that MHV infection was not inducing salmonella resistance by stimulating host killing during the salmonella clearance phase immediately following salmonella injection, the phase during which most salmonella killing occurs. To determine if MHV infection stimulated host killing between salmonella injection and mouse sacrifice 4 days later, MHV-infected and control mice were injected with an Aro$^-$ S. typhimurium mutant that cannot grow in vivo. There was no difference in Aro$^-$ salmonella recovery from the spleens of MHV-infected and control mice four days after salmonella challenge, but there were significantly more salmonella recovered from the livers of MHV-infected mice. This result suggested that MHV infection was not stimulating host killing, but rather was inducing salmonella growth suppression.

MHV-infected and control mice were also injected with a salmonella strain bearing the temperature-sensitive plasmid pHSG422 conferring kanamycin resistance. At 24 and 48 hours after salmonella injection, in spleens and livers both the number of salmonella with the plasmid and the total number of salmonella

were determined using agar with and without kanamycin. In contrast with the results obtained with the Aro⁻ salmonella, no difference in killing between MHV-infected and control mice was found in the liver or spleen by 48 hours after salmonella infection, a result which did, however, support the hypothesis that MHV induced salmonella resistance by growth suppression and not killing. Although the studies as a whole did not determine if inhibition of salmonella killing is a significant effect of MHV infection during the early salmonella clearance phase following salmonella injection, it is apparent that the increased resistance to acute salmonella infection induced by MHV infection is not due to an increase in host killing of salmonella, but rather to growth suppression.

## INTRODUCTION

We have previously documented that when euthymic mice are intranasally infected with a field strain of mouse hepatitis virus (MHV) 6 days before they are intravenously infected with Salmonella typhimurium, the MHV-infected mice have significantly fewer salmonella in spleens and livers 3, 4, and 5 days after salmonella infection than do control mice that receive sterile medium in place of MHV. MHV infection was able to significantly enhance host resistance to salmonella in BALB/cAnNCr mice when salmonella doses up to $10^5$ cfu were used (1). To better understand how MHV infection results in increased salmonella resistance, we wanted to determine if the host was suppressing salmonella growth or more efficiently killing the salmonella. Differentiating between growth suppression and killing is important because different intracellular mechanisms of resistance are probably involved in these two effects, and thus the focus of future investigations of intracellular resistance mechanisms depends on distinguishing

growth suppression from killing. For instance, the generation of oxygen radicals is associated with intracellular macrophage killing (2,3), whereas nutrient sequestration is associated with intracellular growth suppression (4). To determine the effect of MHV infection on in vivo salmonella growth and killing, we used salmonella with the Aro$^-$ mutation that are incapable of in vivo growth, and salmonella bearing a temperature-sensitive plasmid with kanamycin-resistance as a marker to differentially evaluate the kinetics of both growth and killing. The results show that MHV infection did not stimulate the ability of the host to kill salmonella during the initial clearance phase or as late as four days after salmonella injection. We conclude that MHV stimulates mechanisms that suppress salmonella growth.

## RESULTS

### MHV infection inhibits host killing of salmonella in the initial clearance phase immediately following salmonella infection

During the four hour period of time immediately following salmonella injection all of the salmonella are cleared from the blood and about 90% of them are killed (5,6). To determine whether or not MHV infection resulted in a reduction in salmonella numbers in spleens and livers during the early clearance phase immediately following salmonella injection, the number of salmonella in spleens and livers was determined four hours after salmonella infection. To verify that MHV infection did not alter the distribution of salmonella among other tissues and organs, the number of salmonella in the kidneys, lungs, and blood also were determined.

BALB/cAnNCr mice were given either 100 pfu of MHV-UAB or sterile medium intranasally, then $10^6$ virulent S. typhimurium intravenously 6 days later. Four hours after salmonella infection, MHV-infected mice had significantly more salmonella in both spleens and livers than did control mice that received sterile medium in place of MHV. MHV-infected mice had 3 times as many salmonella as control mice and 1.5 times as many in the spleen (Figure 1). Recovery of the salmonella, expressed as a percentage of the initial dose, was <0.5%, <0.05%, and <0.005% in the kidneys, lungs, and blood, respectively in both MHV-infected and control mice. Thus, MHV-infected mice had more salmonella four hours after salmonella infection, and not fewer as is observed 72 hours after salmonella infection (1). This indicated that MHV was not stimulating host killing during the initial clearance phase following salmonella infection.

Use of Aro salmonella to monitor killing by the host

Salmonella with the Aro$^-$ mutation cannot synthesize folate, enterobactin, and some aromatic amino acids and consequently cannot grow in vivo (7). Therefore any reduction in numbers of Aro$^-$ salmonella in vivo is a measure of killing of the salmonella uncomplicated by salmonella growth. As before, BALB/cAnNCr mice were given either 100 pfu of MHV or sterile medium, then 6 days later were injected with salmonella. Paired groups were given $10^5$ or $10^6$ Aro$^-$ salmonella, or $10^4$ or $10^5$ cfu of the Aro$^+$ (wildtype) parental salmonella. Doses of $10^4$ Aro$^-$ and $10^6$ Aro$^+$ salmonella were not used because too few Aro$^-$ salmonella would have been recovered from tissues for accurate cfu determinations, and $10^6$ wildtype salmonella would have killed the mice before the sacrifice time at 4 days post salmonella infection. As expected, MHV-infected mice had significantly fewer Aro$^+$

salmonella in spleens and livers than control mice at both the $10^4$ and $10^5$ doses (Figure 2). In the mice given Aro$^-$salmonella there was no difference in the number of Aro$^-$ salmonella recovered from the spleens of MHV-infected and uninfected mice at either dose, whereas the livers of MHV-infected mice had significantly more Aro$^-$ salmonella at both doses than did those of controls. The difference between the spleens and livers was consistent with the results of the 4 hour organ distribution studies, in which the greatest inhibition in host killing induced by MHV was in the liver.

Use of salmonella bearing a temperature-sensitive plasmid to assess growth and killing in vivo

To assess the effect of MHV infection on growth and killing of salmonella, we used salmonella bearing a temperature-sensitive plasmid, pHSG422, which confers kanamycin resistance. Because the plasmid cannot replicate at mouse body temperature, in vivo growth of the bacteria results in a decrease in the ratio of kanamycin-resistant to kanamycin-sensitive salmonella. Salmonella growth is monitored by determining the total number of salmonella present in organs and host killing is monitored by determining the number of plasmids (kanamycin-resistant salmonella) in organs. Thus, quantifying both kanamycin-resistant and kanamycin-sensitive salmonella allows simultaneous assessment of both salmonella growth and host killing. This technique has been used to provide new insights into the relative contributions of growth suppression and killing in the effects of Ity alleles (8, Benjamin WH Jr, Briles DE submitted).

BALB/cAnNCr mice were given 100 pfu of MHV sterile medium as before, then 6 days later all mice were injected with $10^6$ salmonella with a pHSG422 copy

number of about 1. Twenty-four and 48 hours later, the total number of salmonella and the number of salmonella with the plasmid were determined individually for spleens and livers (Figure 3). At 24 hours, about the same number of total salmonella were recovered from the livers and spleens of MHV-infected and uninfected (control) mice. At 48 hours, a lab error prevented accurate counting of the total salmonella in the organs. In the liver, there was no significant difference between the number of plasmid-bearing salmonella in MHV-infected and control mice at 48 hours. In the spleen, there was a slight though statistically significant decrease in the number of plasmid-bearing salmonella in MHV-infected mice vs. control mice at 24 hours, but no difference at 48 hours. These results indicate that by 48 hours there is no cumulative difference in the amount of salmonella killing between MHV-infected and control mice.

## DISCUSSION

Four hours after injection of salmonella, MHV-infected mice had significantly greater numbers of salmonella in the liver than did control mice not infected with MHV. There were also significantly more salmonella in the spleens of MHV-infected mice, but the magnitude of the difference was less. These results could be explained by enhanced uptake of the organisms rather than decreased killing, but this seems unlikely because there were no differences in the number of salmonella in the blood, lungs, or kidneys, indicating that MHV infection probably does not affect the organ distribution of salmonella after injection. Our finding that over 90% of intravenously injected salmonella are rapidly taken up by the liver and spleen is consistent with the results of previous reports (9,10). The observation that more <u>Aro</u>$^-$ salmonella were recovered from the livers of MHV-infected mice

was consistent with a hypothesis that MHV infection decreased initial host killing. Although the details of the effects in spleens and livers vary, the results of these studies indicate that the increased resistance to salmonella infection induced by MHV infection does not result from increased in vivo killing of salmonella. Therefore, the responsible mechanisms probably act by suppressing salmonella growth.

At 48 hours after salmonella injection there was no difference in the number of plasmid-bearing salmonella between MHV-infected and control mice. Thus, these results also indicate that MHV infection does not affect cumulative host killing of salmonella.

In a previous study (1) we found that nude mice infected with MHV 6 days before salmonella injection were less resistant to salmonella than control mice, rather than more resistant as are immunocompetent mice. In other experiments, we found that in nude mice MHV infection has effects on salmonella killing during the initial clearance phase (up to four hours after injection) similar to those observed in the immunocompetent mice in this study (data not shown). Therefore, it appears that the inhibition of salmonella killing by the host during the initial clearance phase is not a T-dependent mechanism. Figure 4 depicts one possible MHV infection scenario incorporating both early effects on salmonella killing and differences in salmonella growth suppression by the host controlled by the Nu locus.

We hope that by understanding the mechanisms of salmonella resistance induced by MHV we can gain insight into the pathogenesis of systemic salmonella infections as well as how to better control intracellular pathogens in general. It is further hoped that an understanding of how MHV infection results in growth

suppression may be useful in designing viral-based immunostimulatory therapies in patients analogous to the use of Newcastle disease virus infection in the treatment of some neoplasms in human patients (11).

## MATERIAL AND METHODS

Mice. Weanling female BALB/cAnNCr mice from the National Cancer Institute breeding facility at Frederick, Maryland were received in filter-ported crates. Mice were maintained in microisolator cages (Lab Products, Aberdeen, MD) with sterile food, water and bedding. Sera from some mice were screened for antibodies to MHV, Sendai Virus, lymphocytic choriomeningitis virus, mouse rotavirus, pneumonia virus of mice, Reovirus 3, Theiler's GD-VII virus, minute virus of mice, K virus, mouse pox virus, and Mycoplasma pulmonis by Charles Rivers Professional Services (Wilmington, MA) to verify that they were free of these pathogens before experiments began. Mice were used in experiments at 9-16 weeks of age. Experimental groups contained 6 mice, except for the data presented in Figure 1 in which 12 mice per group were used.

Virus. Frozen aliquots (-70°C) of fourth passage mouse hepatitis virus strain UAB (MHV-UAB) were thawed just prior to use in all experiments. This murine coronavirus is a field strain whose isolation has been previously described (12). Experimental mice were anesthetized with an intraperitoneal injection of ketamine (BioMed, San Diego, CA, 100 mg/kg) and rompun (Haver-Lockhardt, Minneapolis, MN, 10 mg/kg), then were inoculated intranasally (IN) with virus (6 days before they were infected with salmonella). Control mice received an equal volume of sterile medium IN in place of MHV.

Salmonella. Salmonella typhimurium strain SR-11 was used in all experiments. For the 4 hour organ distribution and Aro salmonella studies, frozen aliquots (-70°C) of bacteria from late log phase broth cultures were thawed and appropriately diluted in sterile saline just prior to injection. Virulent $Aro^+$ wildtype (parental) salmonella were used in the organ distribution and Aro studies. The SR-11 $Aro^-$ strain also used in the Aro study was prepared by P22 phage transduction. Wildtype SR-11 salmonella were made $Aro^-$ by transduction with a P22 lysate of $Aro^-$ strain SL3235 (7) using procedures described elsewhere (13).

The preparation of SR-11 salmonella bearing the temperature-sensitive plasmid pHSG422 is described in detail elsewhere (Benjamin WH Jr, Briles DE submitted). Briefly, salmonella are maintained in broth at 30°C (permissive temperature) until stationary phase occurs to allow the copy number to peak at 5-8 per cell. Then a portion of the culture is seeded into fresh medium and incubated for about 1.5 hours at 37°C, at which time the copy number has diluted to about 1, the target copy number. The salmonella are then quickly harvested, diluted appropriately in saline, and injected into mice.

Determination of salmonella counts in organs. The number of salmonella cfu in spleens and livers was determined individually. Each organ was homogenized in cold saline with a Stomacher tissue homogenizer (Tekmar Company, Cincinnati, OH), further diluted in cold saline, then plated on Brain-Heart Infusion agar (BBL Microbiology Systems, Cockeysville, MD). To determine the number of salmonella bearing the pHSG422 plasmid, agar was prepared with 5 ug/ml of kanamycin (BBL). With the exception of the kanamycin plates which were incubated at the permissive temperature of 30°C, the plates were incubated at 37°C. The number of salmonella

recovered from organs was expressed as a base ten logarithm. In the organ distribution study the number of salmonella in the lungs and kidneys was determined individually as described above. The number of salmonella in blood was determined by directly plating the neat and saline dilutions of blood on agar.

Statistical analysis. Data were analyzed by computer (Statistix II software package, NH Analytical Software, Roseville, MN) using the multifactorial analysis of variance (ANOVA) technique. Differences between group geometric means were considered to be significantly different when p was less than 0.05. In the event of significant factor interactions ($p<0.05$), differences between individual means were tested for significance with Scheffe's test (14).

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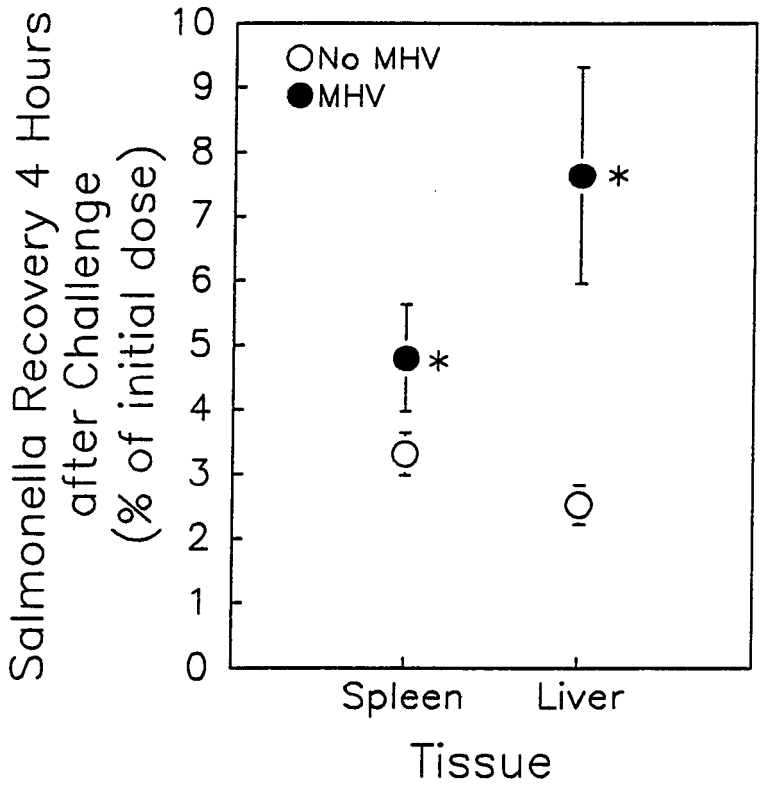
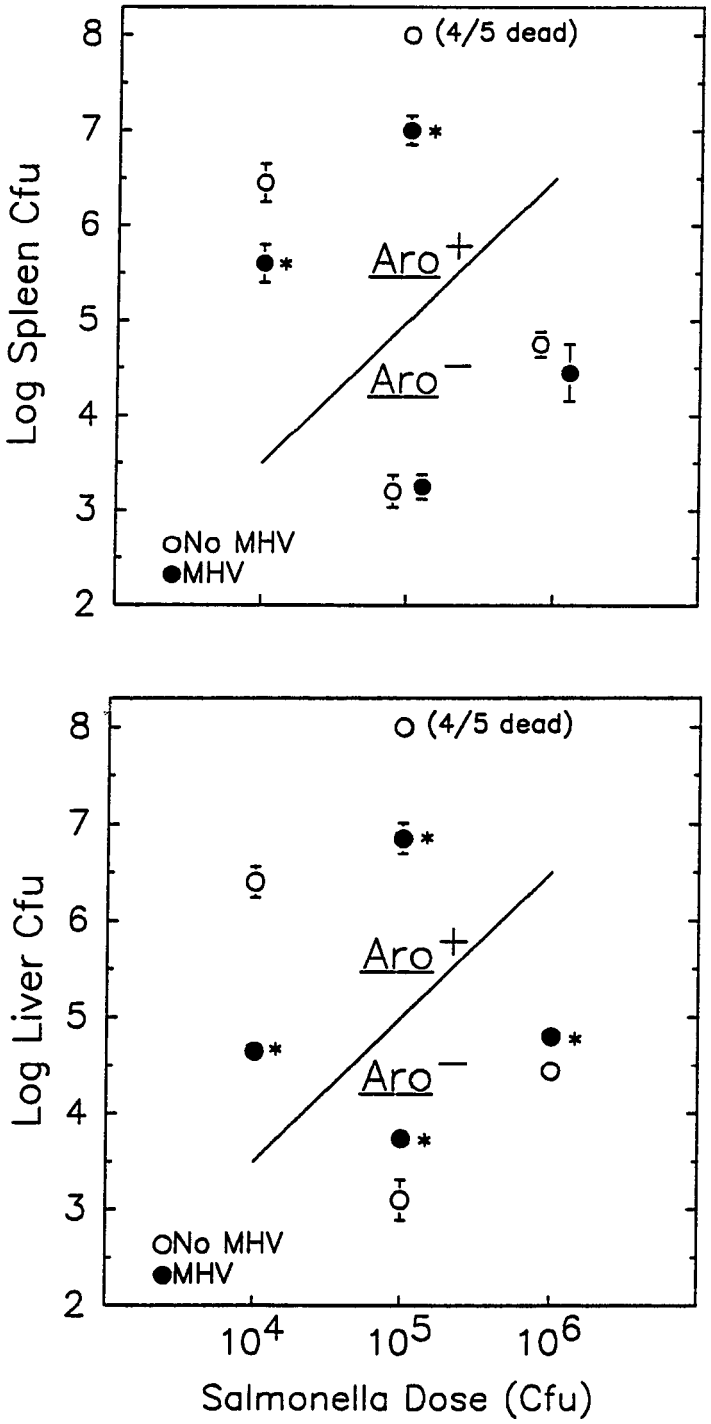



**Figure 1.** Effect of MHV infection on salmonella distribution in spleens and livers 4 hours after salmonella injection. Mice received either 100 pfu of MHV (closed circles) or sterile medium (open circles), then 5 $x10^5$ salmonella cfu intravenously 6 days later. Symbols represent the geometric means (+/- SE) of the number of salmonella recovered from organs 4 hours after salmonella infection. Asterisks indicate significant differences between MHV-infected and uninfected groups at $p<0.05$.

Figure 2. Effect of MHV infection on recovery of Aro$^+$ and Aro$^-$ salmonella from spleens and livers. Mice received either 100 pfu of MHV (closed circles) or sterile medium (open circles), then the number of salmonella cfu's shown on the x-axis 6 days later. Symbols represent the geometric means (+/- SE) of the number of salmonella recovered from spleens (a) and livers (b) 4 days after salmonella infection (the symbols sometimes hide the error bars). Asterisks indicate significant differences between MHV-infected and uninfected groups at $p<0.05$.

**FIGURE 2**


Log Spleen Cfu
8
7
6
5
4
3
2
o (4/5 dead)
Aro+
Aro−
oNo MHV
●MHV
Log Liver Cfu
8
7
6
5
4
3
2
o (4/5 dead)
Aro+
Aro−
oNo MHV
●MHV
10^4
10^5
10^6
Salmonella Dose (Cfu)

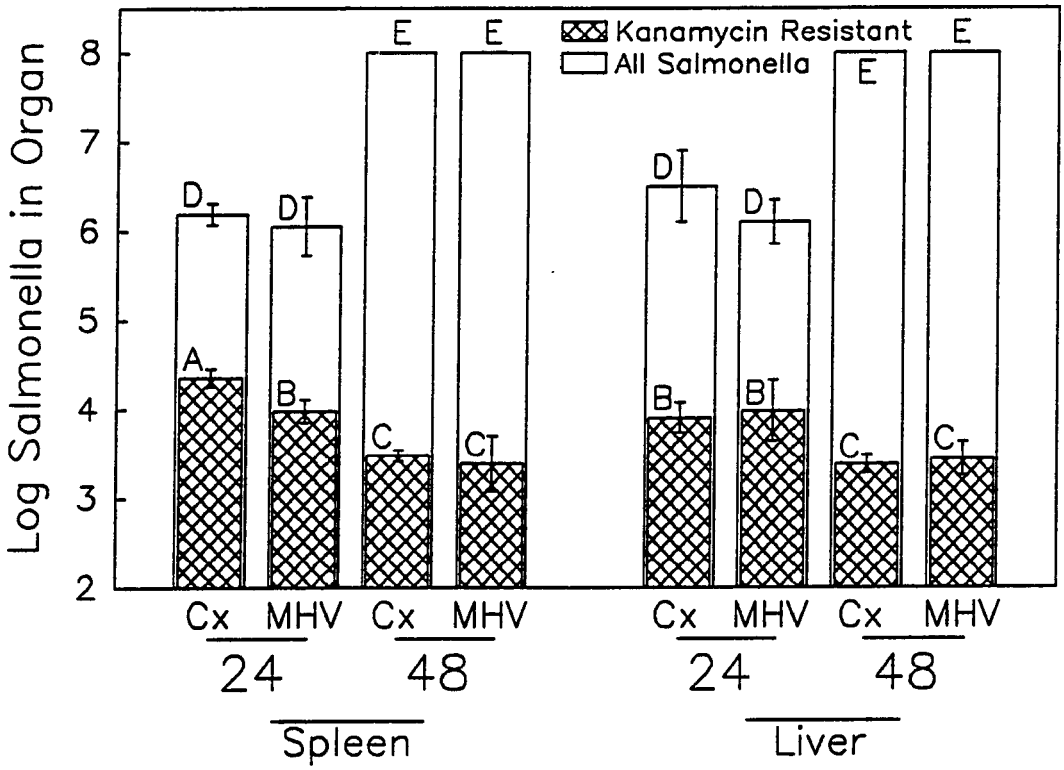



**Figure 3.** Effect of MHV infection on recovery of salmonella bearing the pHSG422 temperature-sensitive plasmid. Mice received either 100 pfu of MHV or sterile medium, then 1 $x10^6$ salmonella with a plasmid copy number of 0.8 intravenously 6 days later. Bars represent the geometric means (+/- SE) of the number of salmonella recovered from organs 24 or 48 hours after salmonella infection. The open bars represents the total number of salmonella recovered from the organ and the cross-hatched bars represent the number of kanamycin resistant plasmid-bearing salmonella recovered. Asterisks indicate significant differences between MHV-infected and uninfected groups at $p<0.05$.

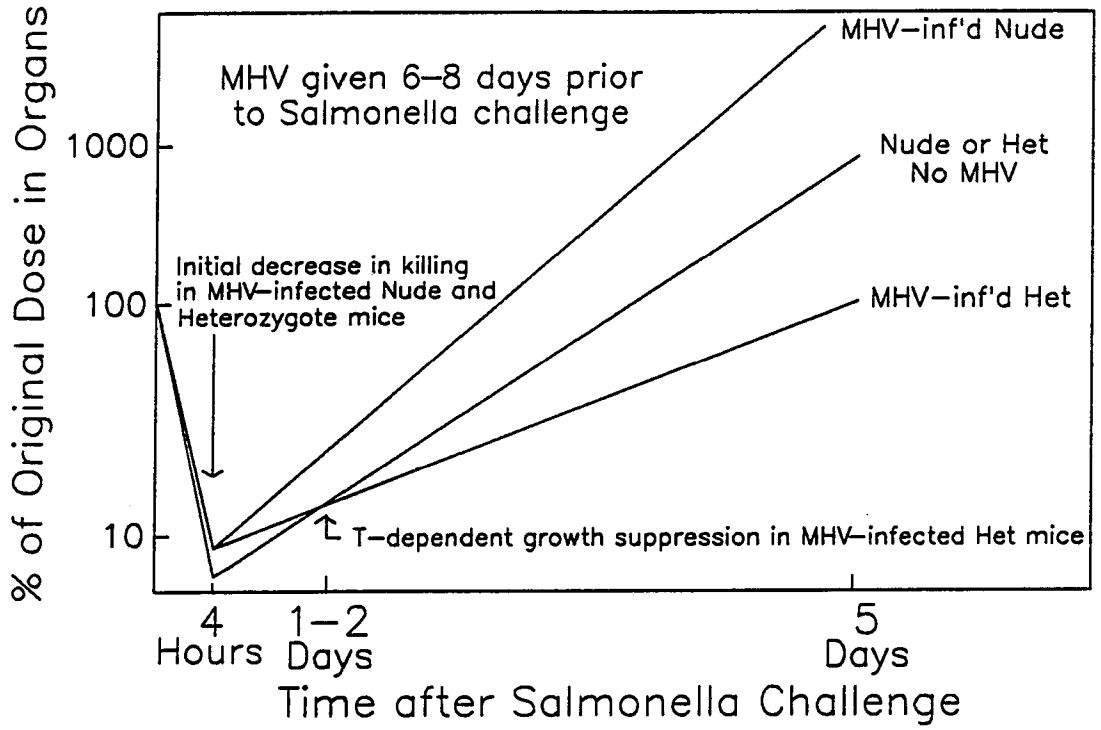



**Figure 4. Unified hypothesis of MHV-mediated effects on salmonella resistance. The solid line indicates the expected kinetics of salmonella killing and growth following intravenous challenge in BALB/c-nu/+ (heterozygote) or -nu/nu (nude) mice not infected with MHV. The dotted lines indicate the expected kinetics of salmonella killing and growth in MHV-infected heterozygote or nude mice when MHV is given 6 days before salmonella.**

Having established that MHV infection induced salmonella resistance in euthymic mice by growth suppression of the bacteria by the host, the question of cellular mechanisms became important. Pilot experiments indicated that large amounts of type I interferon (IFN) was released by mice following MHV-UAB infection. Type I IFN is a classic mediator released by leukocytes and fibroblasts in response to many viral infections. The following manuscript concerns an investigation of the role of type I IFN in the modulation of salmonella resistance by MHV-UAB.

MANUSCRIPT #3

# ROLE OF TYPE I INTERFERON IN THE MODULATION OF RESISTANCE TO SALMONELLA TYPHIMURIUM IN MICE BY MOUSE HEPATITIS VIRUS (MHV) AND POLY I:C

By

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Prepared for submission to Infection and Immunity

## ABSTRACT

Prior infection of euthymic mice with MHV strain UAB results in increased resistance to virulent Salmonella typhimurium infection when the interval between MHV and salmonella infections is 6 to 8 days. Studies were undertaken to evaluate the role of interferon (IFN) production by MHV-UAB in the induction of salmonella resistance in mice. No gamma-IFN could be detected in spleen supernatants or serum of MHV-infected BALB/cAnNCr mice, but large amounts of type I (alpha/beta) IFN were found. The potent type I IFN inducer poly I:C was able to qualitatively mimic the effects of MHV on host resistance to salmonella, although the kinetics of IFN release after poly I:C treatment were not the same as that during MHV infection. Direct evidence that type I IFN at least partially mediated the induction of salmonella resistance by MHV was provided by the observation that rabbit immune serum that neutralized type I mouse IFN was able to significantly inhibit the induction of resistance to salmonella by MHV infection. We concluded that induction of type I IFN by MHV infection did contribute to the induction of salmonella resistance by MHV infection. We also found that salmonella infection alone produced modest amounts of type I IFN. Paradoxically, administration of the IFN neutralizing antiserum to mice not infected with MHV caused an increase in resistance to salmonella. This indicates that the host's IFN response may be beneficial or detrimental to salmonella resistance, depending on the presence of other, as yet unidentified, factors; MHV infection therefore must have other effects on the host which interact with IFN to induce resistance to salmonella.

## INTRODUCTION

In studies of the effects of mouse hepatitis virus (MHV) infection on host resistance to Salmonella typhimurium in the mouse, we established that infection of euthymic mice with MHV strain UAB (MHV-UAB) could enhance the resistance of mice to intravenous challenge with virulent S. typhimurium (8). Initial studies of MHV-UAB showed the virus to be a potent inducer of type I (non-immune) interferon (IFN); therefore we conducted experiments to determine whether or not IFN mediates the increased resistance to salmonella following MHV infection. We found that the potent type I IFN inducer polyinosinic:polycytidilic acid (poly I:C) (5) could qualitatively mimic the effects of MHV on salmonella infection, and that anti-type I neutralizing antibody could significantly reduce the ability of MHV to induce salmonella resistance. However, antiserum to type I IFN given to mice not inoculated with MHV increased resistance to salmonella, indicating that MHV infection acts on salmonella resistance through other mechanisms which interact with IFN.

## MATERIALS AND METHODS

Mice. Weanling female BALB/cAnNCr and A/JCr mice from the National Cancer Institute breeding facility at Frederick, Maryland were received in filter-ported crates. Mice were maintained in microisolator cages (Lab Products, Aberdeen, MD) with sterile food, water, and bedding. Sera from some mice were screened for antibodies to MHV, Sendai Virus, lymphocytic choriomeningitis virus, mouse rotavirus, pneumonia virus of mice, Reovirus 3, Theiler's GD-VII virus, minute virus of mice, K virus, mouse pox virus, and Mycoplasma pulmonis by Charles Rivers Professional Services (Wilmington, MA) to verify that they were

free of these pathogens before experiments began. Mice were used in experiments at 9-16 weeks of age.

Virus. Frozen aliquots (-70°C) of fourth passage mouse hepatitis virus strain UAB (MHV-UAB) were thawed just prior to use in all experiments. This murine coronavirus is a field strain whose isolation has been previously described (4). Experimental mice were anesthetized with an intraperitoneal injection of ketamine (BioMed, San Diego, CA, 100 mg/kg) and xylazine (Haver-Lockhardt, Minneapolis, MN, 10 mg/kg), then were inoculated intranasally (IN) with virus (6 days before they were infected with salmonella). Control mice received an equal volume of sterile medium IN in place of MHV.

Poly I:C injection. Commercial poly I:C (Sigma, St. Louis, MO) was purchased in lyophilized form and rehydrated with pyrogen-free saline. Aliquots of the solution (0.5 ug poly I:C per ul) were kept frozen at -70°C until used. Mice were injected intraperitoneally with the poly I:C solution immediately after it thawed.

Salmonella. Virulent Salmonella typhimurium strain SR-11 was used in all experiments. To ensure reproducible dosing, frozen aliquots (-70°C) of bacteria from late log phase broth cultures were thawed and appropriately diluted in sterile saline just prior to injection in mice via a lateral tail vein.

Determination of salmonella counts in organs. The number of salmonella cfu in spleens and livers was determined individually. Each organ was homogenized in cold saline with a Stomacher tissue homogenizer (Tekmar Company, Cincinnati, OH), further diluted in cold saline, then plated on Brain-Heart Infusion agar (BBL Microbiology Systems, Cockeysville, MD) and incubated overnight at 37°C. The

number of salmonella recovered from organs was expressed as a base ten logarithm.

Collection of samples for analysis of IFN. Spleen cell supernatants and serum were collected individually from mice for analysis of interferon content. Mice were anesthetized with ketamine and xylazine by the intraperitoneal route, then were bled out from a cut in an axillary region. Blood was allowed to clot for 30 minutes, then was spun down in a microcentrifuge for 3 minutes. Serum was collected, diluted 1:3 in cold phosphate-buffered saline (PBS) that had been autoclaved at 250°C for 4 hours to remove endotoxin, then passed through a low protein binding filter. The filtrate was stored at -70°C until it could be assayed for interferon. Spleens were removed from mice and placed into autoclaved nylon mesh boats in small Petri dishes containing cold PBS. After the pulp had been forced through the mesh, the spleen cell suspension was sonicated for 15 seconds to disrupt the cells. Cell debris was removed by centrifugation in a refrigerated centrifuge at 500g. The supernatant was collected and pushed through a low protein binding 0.22 um syringe filter and stored at -70°C until it also could be assayed for interferon (1 ml final volume per spleen).

Interferon assay. IFN was assayed as previously described (16). Briefly, three-fold dilutions of the test samples were incubated with mouse L929 cell monolayers for 24 hours in 96 well plates. After successive washes, about 50 pfu of vesicular stomatitis virus was added to each well. Four hours later, the cells were washed again and overlayed with medium containing 0.5% agar. After 48 hours, the cells were washed and fixed with a 4% crystal violet-ethanol mixture. After drying, the wells were counted. The IFN titer was defined as the reciprocal of the highest dilution of sample that inhibited plaque formation by 50%. An

internal IFN standard verified the accuracy of the assay. The detection limits using this assay were about 60 units of IFN per ml of serum and 20 units of IFN per spleen.

To type the IFN in the samples, selective neutralization of IFN activity by polyclonal rabbit antiserum specific for mouse type I and type II IFN was performed (17). Purified type I and type II IFN was included in these neutralization assays to verify the validity of the typing system.

Use of interferon neutralizing antibody in vivo. Rabbit polyclonal anti-mouse alpha/beta IFN serum was purchased in lyophilized from Lee Biomolecular (San Diego, CA). It was rehydrated with pyrogen-free sterile water according to the package insert. Mice were given 5000 neutralizing units of serum in 100 ul volume by the intraperitoneal route. Control mice equal volumes of normal rabbit serum. The antiserum was given 15 minutes before either MHV infection or poly I:C injection.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA). Treatment effects were considered to be significantly different when p was less than or equal to 0.05. Differences between pairs of individual group means were tested for significance with Scheffe's test (3).

## RESULTS

IFN release following MHV and salmonella infections. To determine whether or not IFN was produced by MHV-UAB or salmonella infection, 24 mice were given 100 pfu of MHV, then spleen supernatant and serum samples were obtained when groups of 4 mice were sacrificed 1, 3, 5.5, 6.5, 8, and 10 days later. Those mice not sacrificed by day 6 were also injected with 4000 cfu of salmonella on day 6.

An additional 24 control mice were treated identically except they received sterile medium in place of MHV. The numbers of salmonella in the livers of MHV-infected and uninfected (control) mice that received salmonella were determined individually to determine the relationship between the salmonella burden and the amount of IFN in serum and spleen. One day after MHV infection over 30,000 units of IFN were detected per ml of serum vs. about 600 units in the total spleen. By the 3rd and 6th days post MHV infection the levels of IFN in the serum and spleen, respectively, returned to baseline (Figure 1A). The amount of IFN recovered after salmonella injection from the spleens of both MHV-infected and control mice were roughly proportional to the number of salmonella in the liver, which consistently contains about the same number of salmonella as the spleen (1) (Figure 1B). All IFN detected was typed as alpha/beta IFN. Thus, it was established that MHV-UAB infection was a potent inducer of alpha/beta IFN, and that in the absence of MHV infection there was also an alpha/beta IFN release in the spleen proportional to its salmonella burden.

Dose response of IFN release to MHV infection and poly I:C injection. To determine the dose response relationship between MHV dose and IFN production, groups of mice were given 100, 50, 5, 0.5, or 0.05 pfu of MHV intranasally, or 150, 100, or 50 ug of poly I:C intraperitoneally. Poly I:C is a double stranded polynucleotide that potently induces alpha/beta IFN (10,11). An additional group received no treatment. Mice from each group were sacrificed 0.5, 3, or 6 days later and the amount of IFN in the serum was determined. There was a direct relationship between the height of the IFN response curves and the poly I:C dose (Figure 2). In contrast, there was an inverse relationship between the height of the

IFN curves and the MHV dose with the exception of the mice that received the 0.05 pfu MHV dose, which had the smallest IFN response curve. The kinetics of IFN release induced by MHV and poly I:C were different; the response to poly I:C rose and fell more rapidly than that to MHV infection. This may be because poly I:C can be processed by cells to act as an IFN inducer immediately after injection, whereas some replication of the virus may be required before much IFN is induced.

To determine whether or not the magnitude of salmonella resistance induced by MHV infection was proportional to the amount of alpha/beta IFN released by virus infection, additional experimental groups were included in the above experiments. Groups of mice were given 50, 5, 0.5, 0.05, or 0 (control) pfu of MHV as above, but were injected with 10,000 cfu of salmonella 6 days after MHV infection. Four days after salmonella injection, the mice were sacrificed and the number of salmonella in their spleens and livers was determined. As expected, the MHV-infected mice had significantly fewer salmonella in both spleens and livers than did control mice ($p<0.05$). The number of salmonella recovered from the spleens of the mice decreased linearly as the MHV dose increased (Figure 3, spleen data shown), and the number of salmonella recovered from the 50 pfu dose group was significantly less than that recovered from the 0.05 pfu mice ($p<0.05$). However, as seen in Figure 2, the amount of IFN produced in the serum was not proportional to the MHV dose (3 day post MHV data is presented again in Figure 3 for comparison), suggesting that the magnitude of the salmonella resistance induced by IFN was not due directly to the amount of IFN released following MHV infection. This result did not, however, rule out a role of alpha/beta IFN in the induction of salmonella resistance by MHV.

Substitution of poly I:C for MHV. To determine whether or not another type I IFN inducer could mimic the affects on salmonella resistance obtained with MHV infection, 100 ug of poly I:C or 100 pfu of MHV was given to groups of A/JCr and BALB/cAnNCr mice, then 1, 3, 6, or 8 days later 20,000 cfu of salmonella was injected into all mice. All mice were sacrificed 4 days post salmonella challenge and the number of salmonella in their spleens and livers was determined. Since the spleen and liver data were essentially identical in nature, only the spleen data is presented. The number of salmonella recovered from the treatment groups was compared with the number of salmonella in the control mice of each mouse strain which received no treatment. In the BALB/cAnNCr mice MHV infection did not alter salmonella resistance at an MHV-salmonella interval of 3 days, but MHV-infected mice had significantly more salmonella if the interval was 1 day and significantly increased salmonella resistance if the interval was 6 or 8 days (Figure 4A). The same results were obtained with poly I:C injection at the 1, 3, and 6 day poly I:C-salmonella intervals, but poly I:C injection did not affect salmonella recovery at the 8 day interval. Similar effects, although lesser in magnitude, were observed in A/JCr mice (Figure 4B) which are more resistant to salmonella as a result of their Ity$^r$ genotype (1). MHV-infected A/JCr mice had significantly more salmonella at the 1 or 3 day MHV-salmonella intervals and significantly fewer salmonella at the 8 day interval. There was no difference between MHV-infected and control mice at the 6 day interval. A/JCr mice injected with poly I:C had about the same number of salmonella as did control mice at all intervals except the 1 day interval, at which mice injected with poly I:C had significantly more salmonella than did controls. Although MHV infection and poly I:C injection did

not have exactly the same effect on salmonella resistance in either the BALB/cAnNCr or A/JCr mice, it was apparent that injection of the IFN-inducer poly I:C caused effects on salmonella resistance qualitatively similar to those caused by MHV infection.

Use of IFN neutralizing antibody. To determine whether or not neutralization of alpha/beta IFN in vivo could affect the ability of MHV and poly I:C to induce salmonella resistance, BALB/cAnNCr mice were injected with 5000 neutralizing units of polyclonal rabbit anti-mouse alpha/beta IFN, then 15 minutes later were infected with 100 pfu of MHV or injected with 100 ug of poly I:C. Another group of mice received the antisera but neither MHV nor poly I:C. Three identical groups received an equal volume of normal rabbit serum in place of the anti-IFN antiserum. Six days after experimental treatments, all mice were injected with 20,000 salmonella cfu, then 4 days later all mice were sacrificed and the number of salmonella in spleens and livers was determined.

Neutralization of IFN with antisera resulted in significantly fewer salmonella in the spleens of mice that received neither MHV nor poly I:C, but significantly more salmonella in the spleens of mice treated with MHV and poly I:C (Figure 5A). The same qualitative changes were present in the liver, but only in conjunction with MHV treatment did the neutralizing antibody have a significant effect (Figure 5B). As expected, mice treated with MHV and poly I:C that were given normal rabbit serum had significantly fewer salmonella in both spleens and livers than mice given neither MHV nor poly I:C.

# DISCUSSION

We conclude that type I (alpha/beta) IFN probably contributes to the induction of salmonella resistance by MHV infection. The qualitative similarity of the effect of the type I IFN inducer poly I:C to that of MHV infection on resistance to salmonella (Figure 4) provided indirect evidence of a role for type I IFN in MHV-mediated induction of salmonella resistance. Poly I:C undoubtedly causes the release of many mediators, and it is known to have effects on the host different from those caused by exogenously administered IFN. For instance, unlike IFN, poly I:C enhances antibody production (10). The effects of poly I:C on salmonella resistance usually were less strong than those of MHV. A higher dose of poly I:C might have given greater biological effects, but the dose used is known to stimulate NK cells in mice in vivo (5,11) and it produced IFN levels about equal in magnitude to those following inoculation with 100 pfu of MHV (Figure 2). The decreased magnitude of poly I:C effects might also be explained by differences in IFN release kinetics (Figure 2) or differences in total IFN production.

To evaluate the importance of IFN in the induction of salmonella resistance by MHV infection, we chose to block IFN with specific antibody rather than to give exogenous IFN by injection, because it is difficult to know if exogenously administered IFN reaches appropriate physiologic spaces in the proper amount. The ability of anti-type I IFN antibody to significantly inhibit the ability of MHV infection to induce salmonella resistance was direct evidence that type I IFN was involved in the induction of salmonella resistance by MHV infection (Figure 5). The inability of the neutralizing antibody to completely block the induction of resistance to salmonella by MHV infection could be due to too little antibody, but

according to the vendor the dose of antibody used was able to neutralize between 3.5 and 5.0 x $10^5$ units of IFN, an amount of IFN approximately equal to the highest amount of IFN detected in spleens or sera following infection with 100 pfu of MHV. It seems likely that other mediators, such as tumor necrosis factor, interact with IFN to stimulate host antibacterial defenses (9). Type I IFN could enhance salmonella resistance indirectly by initiating the release of other mediators, but it could function directly by enhancing NK cell activity (5) or by stimulating macrophages (18). MHV infection is known to both enhance NK cell activity (12) and stimulate macrophages (2), but it is not known if these effects are mediated by IFN.

It is interesting that neutralizing antibody given to untreated mice caused a reduction in the number of salmonella recovered from spleens and livers (Figure 5). Because the spleens and sera of mice inoculated with salmonella but not MHV contained significant amounts of IFN (Figure 1), IFN may be detrimental to salmonella resistance under some circumstances. It may be that IFN inhibits the ability of macrophages to limit intracellular growth of salmonella. Previously it has been reported that gamma IFN enhances the growth of Mycobacterium tuberculosis and M. avium in macrophages (6). If so, the ability of the IFN neutralizing antibody to partially block the induction of salmonella resistance by MHV and poly I:C may have been be due to indirect modulation of mediators other than IFN by the neutralizing antibody.

Although published reports have come to widely divergent conclusions concerning the in vivo ability of MHV strains to cause IFN production (12-15), the MHV-UAB field strain used in these studies is a potent type I IFN producer. The

production of IFN by MHV-UAB may explain the suppression of antibody production by Peyer's patch cells from mice infected with MHV-UAB (4), since it has been established that IFN can inhibit antibody responses (7). It is important to note that our inability to detect gamma IFN does not mean that local release of small amounts of gamma IFN by T cells in areas of inflammation may not be an important aspect of host resistance to salmonella.

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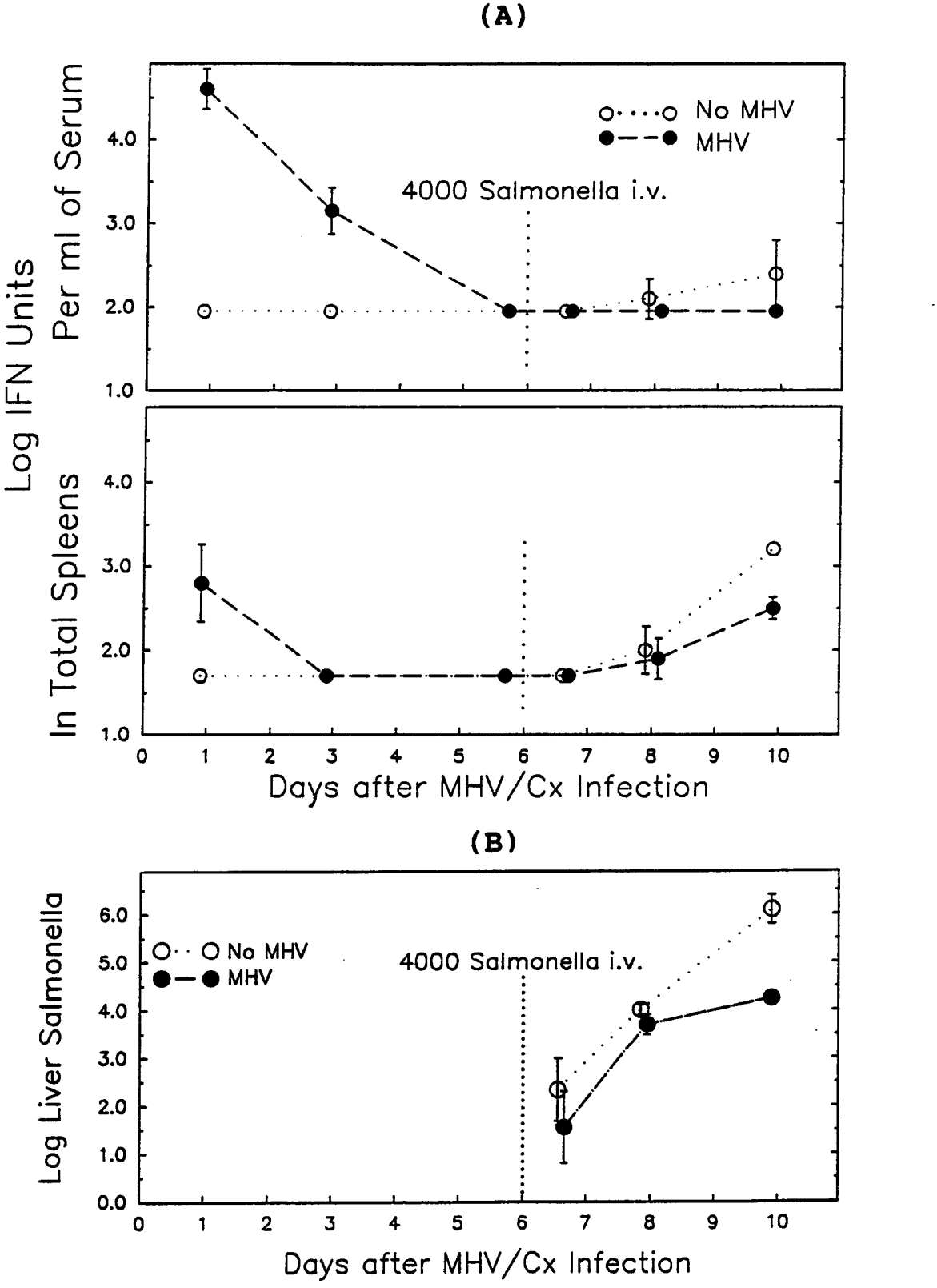
Figure 1. Induction of IFN by MHV and salmonella. Mice were given either 100 pfu of MHV (closed circles) or sterile medium (open circles) on day 0. All mice not yet sacrificed were given 4000 salmonella cfu on day 6. The amount of IFN detected in serum and spleens is shown in (A), and the number of salmonella recovered from the livers of mice infected with salmonella and sacrificed as indicated is shown in (B). Symbols represent the geometric mean +/- the standard error of the mean, 4 mice per group (the symbols sometimes hide the error bars).

**FIGURE 1**


(A)
Log IFN Units
Per ml of Serum
In Total Spleens
No MHV
MHV
4000 Salmonella i.v.
4.0
3.0
2.0
1.0
0 1 2 3 4 5 6 7 8 9 10
Days after MHV/Cx Infection
(B)
Log Liver Salmonella
6.0
5.0
4.0
3.0
2.0
1.0
0.0
No MHV
MHV
4000 Salmonella i.v.
0 1 2 3 4 5 6 7 8 9 10
Days after MHV/Cx Infection

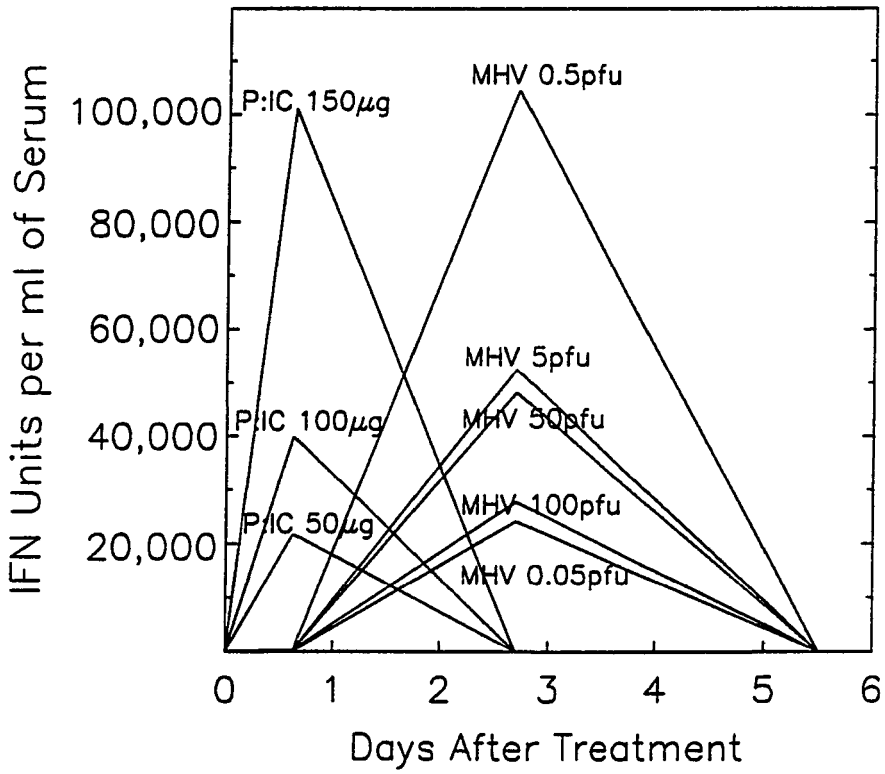



**Figure 2. Kinetics of IFN production following different MHV and poly I:C doses. Mice were given the indicated MHV or poly I:C dose on day 0, then the amount of IFN in serum was determined periodically as shown. IFN amounts were plotted on a linear scale to allow better visualization of IFN dose-response relationships.**

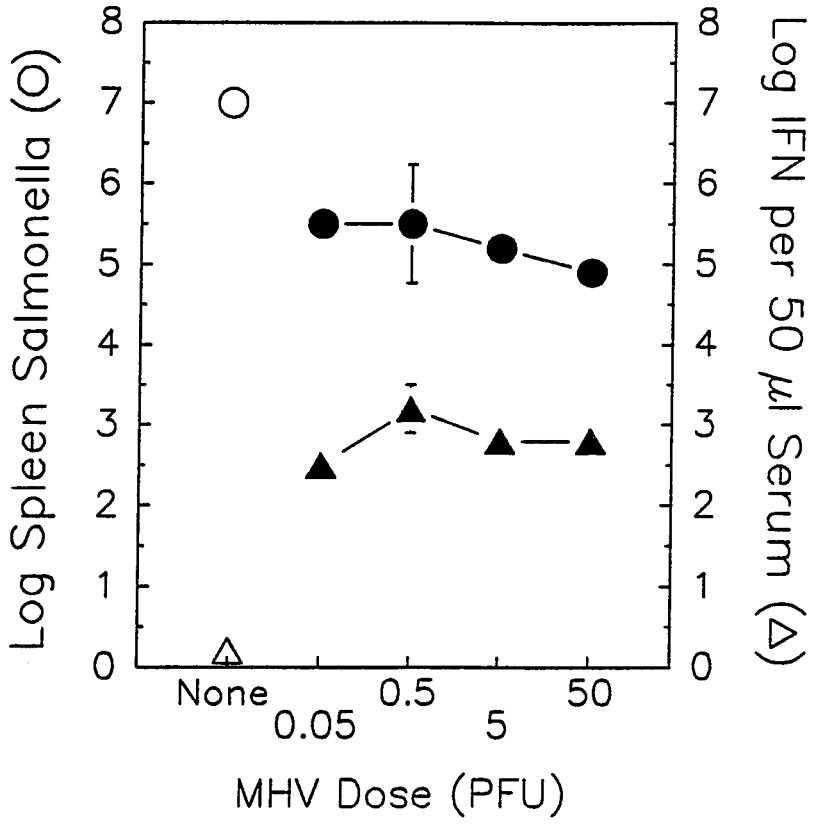



Figure 3. IFN production by MHV and induction of salmonella resistance. Mice were given the indicated doses of MHV (closed symbols) or sterile medium (open symbols). Some groups of mice were injected with 10,000 salmonella cfu 6 days later and the number of salmonella in their spleens was determined (circles). Other groups of mice were sacrificed at various times after MHV infection and the amount of IFN in their serum was determined (triangles) (see Figure 2 for complete serum data). IFN data shown is from mice sacrificed about 3 days after MHV infection (or in the case of controls, inoculation with sterile medium). Symbols represent the geometric mean +/- the standard error of the mean, 4 mice per data point (symbols hide some error bars).

Figure 4. Effect of MHV-salmonella and poly I:C-salmonella interval on salmonella resistance. Mice were given no treatment (open circle), 100 pfu of MHV (closed circles) or 100 ug of poly I:C (open squares), then 20,000 cfu of salmonella 1, 3, 6, or 8 days later. All mice were sacrificed 4 days after salmonella infection. Symbols represent the geometric mean +/- the standard error of the mean, 6 mice per data point (symbols cover some error bars). Groups that were significantly different from the control group are marked with an asterix ($p<0.05$).

**FIGURE 4**

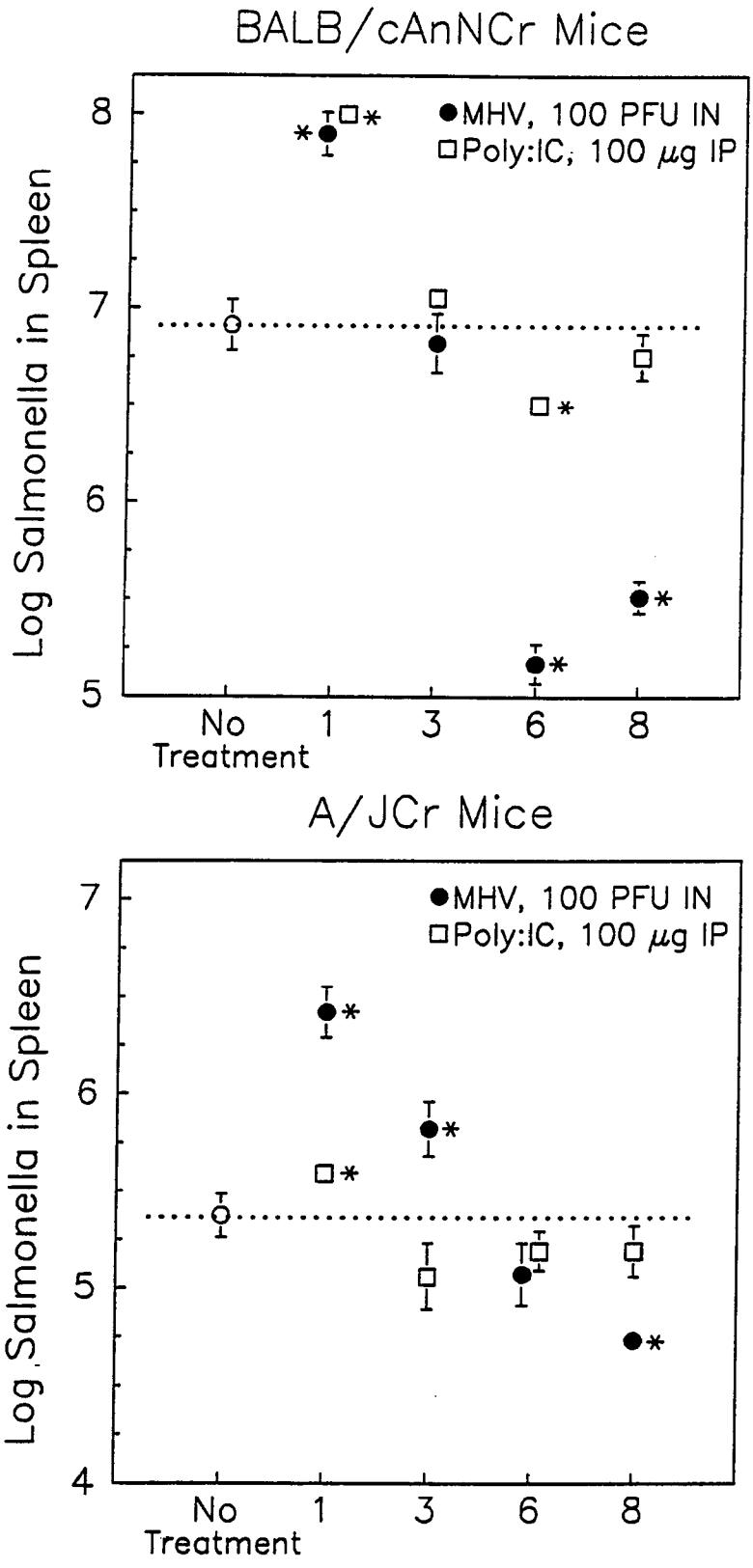
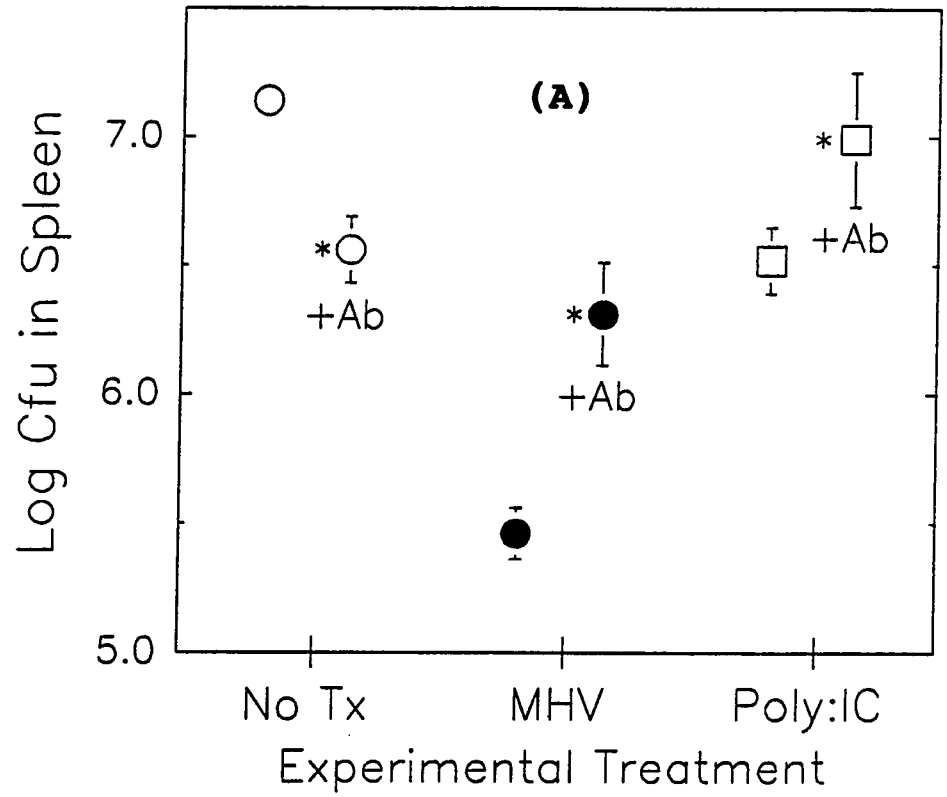
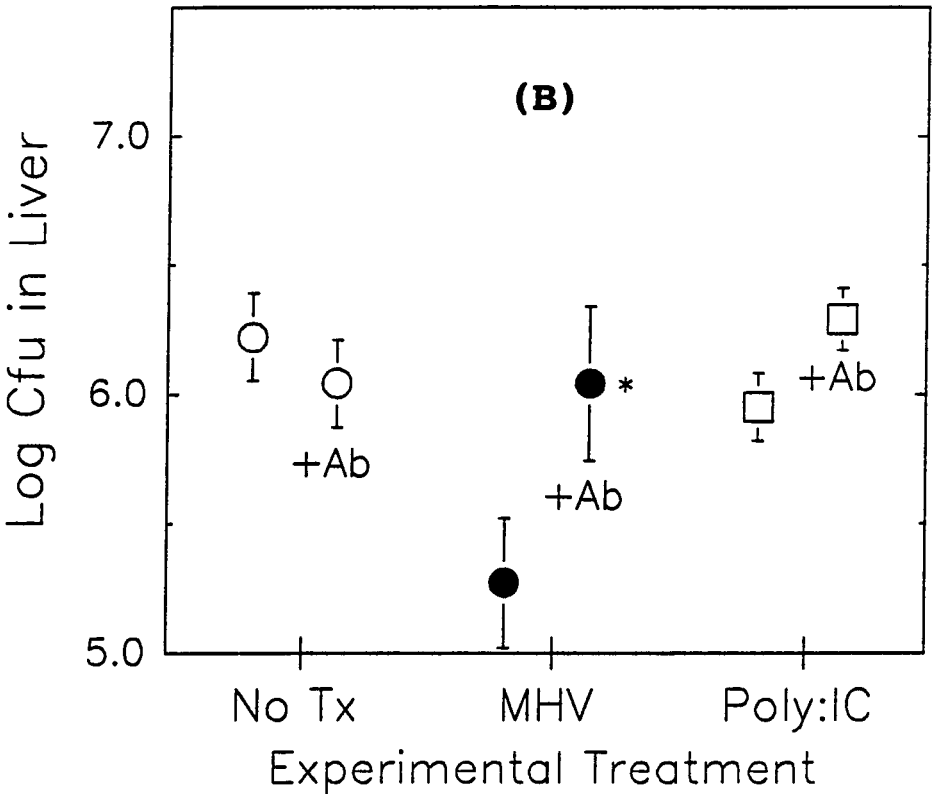

BALB/cAnNCr Mice
Log Salmonella in Spleen
8
7
6
5
●MHV, 100 PFU IN
□Poly:IC, 100 μg IP
No Treatment
1
3
6
8
A/JCr Mice
Log Salmonella in Spleen
7
6
5
4
●MHV, 100 PFU IN
□Poly:IC, 100 μg IP
No Treatment
1
3
6
8
Days Between Treatment and Salmonella Infection

Figure 5. Effect of anti-IFN neutralizing antibody. Paired groups of mice received 100 pfu of MHV (closed circles), 100 ug of poly I:C (open boxes), or neither MHV nor poly I:C (open circles). One group of each pair received 50 ul of normal rabbit serum, the other received 50 ul of rabbit serum containing 5000 neutralizing units of anti-mouse IFN antibody (denoted by "+Ab"). Six days after treatment all mice received 20,000 salmonella cfu, then 4 days later, all mice were sacrificed and the number of salmonella in spleens (A) and livers (B) was determined. Symbols represent the geometric mean +/- the standard error of the mean, 6 mice per data point (symbols cover some error bars). Groups treated with neutralizing antibody that were significantly different from those treated with normal rabbit serum are marked with an asterix ($\underline{p}<0.05$).

**FIGURE 5**


(A)
Log Cfu in Spleen
7.0
6.0
5.0
+Ab
+Ab
+Ab
No Tx
MHV
Poly:IC
Experimental Treatment


(B)
Log Cfu in Liver
7.0
6.0
5.0
+Ab
+Ab
+Ab
No Tx
MHV
Poly:IC
Experimental Treatment

## GENERAL DISCUSSION

Results of these studies clearly show that MHV infection is able to significantly modulate resistance to Salmonella typhimurium infection. In euthymic mice, the interval between MHV and salmonella inoculations determines whether MHV infection increases or decreases resistance to salmonella; at intervals of one to three days between MHV and salmonella inoculations, MHV infection causes decreased resistance to salmonella, whereas at intervals of six to eight days, MHV infection causes increased resistance to salmonella. In contrast, MHV infection of nude mice results in decreased resistance to salmonella when the interval between MHV and salmonella inoculations is six days. This suggests that the resistance mechanisms induced by MHV infection in euthymic mice are T-dependent. This is a significant finding, because T cells are not thought to play an important role in salmonella resistance; transfer of immune T cells has not transferred resistance (13,26,29), and nude mice are no more susceptible to salmonellosis than normal mice until 10 to 14 days after salmonella injection (41, Manuscript #1- Figure 6). On the other hand, sensitized T cells maintained and expanded in vitro with interleukin-2 could transfer resistance to salmonella (45). Also, an alternative explanantion for the lack of increased susceptibility to salmonella infection by nude mice is that they can compensate for their lack of T-dependent salmonella resistance mechanisms with enhanced NK cell and macrophage function (54).

It is interesting that both MHV infection and the ity$^r$ gene mediate resistance to salmonella infection primarily by growth suppression, and not by increased killing (6,9). Because much higher doses of salmonella have to be given to Ity$^r$ mice than Ity$^s$ mice to observe an enhancement of salmonella resistance by MHV infection, rapid salmonella growth may be necessary for the expression of resistance mechanisms induced by MHV infection. Thus, in Ity$^r$ mice where salmonella growth suppression occurs naturally, large doses of salmonella might be required to overwhelm the growth suppression conferred by the ity$^r$ gene. This interpretation is consistent with the results of the studies in which MHV infection did not result in the recovery of fewer non-growing Aro$^-$ salmonella from the spleens or livers of mice.

Our finding that MHV infection inhibits salmonella killing during the initial clearance phase, then causes growth suppression by the fourth day after salmonella injection, is consistent with current theories of host resistance to salmonella. Extensive studies of salmonella resistance using the mouse typhoid model have established that host resistance is mediated by killing in a very brief initial clearance phase, then later by suppression of the growth of surviving bacteria. Surviving salmonella presumably reside in a site that protects them from further killing. The location of this site is uncertain, but it probably is within macrophages (27). Further killing by the host does not occur until seven to ten days post salmonella challenge, when immune-mediated mechanisms can result mediate killing, at least in resistant mice (41).

Based on the results of studies in which neutralizing antibody to type I interferon (IFN) significantly inhibited the ability of MHV to induce resistance to

salmonella, we concluded that type I IFN plays a role in the induction of salmonella resistance by MHV infection. However, in the absence of MHV infection, mice treated with the neutralizing antibody had significantly fewer, rather than more, salmonella, and we also found that a significant type I IFN release occurs following inoculation with salmonella alone. Thus, we conclude that IFN release by the host in response to salmonella infection is not necessarily beneficial to the host. Furthermore, these results suggest that MHV infection induces other host alterations that interact with IFN to induce salmonella resistance. It is interesting to note that when macrophages infected with mycobacteria are treated with gamma IFN, the macrophages are less able to limit the growth of intracellular mycobacteria (17). It is interesting to note that the Ity locus also controls the resistance of mice to some mycobacteria (41). Thus, the Ity locus may influence salmonella resistance by controlling either the amount of IFN released during salmonella infection or the sensitivity of macrophages to IFN.

Many additional questions were raised by results of these studies. It is unclear whether or not T cells are necessary for induction of salmonella resistance by MHV or for resistance of naive mice to salmonella, in part because nude mice are not simply normal mice that lack T cells. For example, they may have higher levels of NK cell and macrophage activity than normal mice. Therefore, many of the assumptions based on experiments in nude mice about the role of T cells in infectious diseases may be misleading. The role of T cells could be addressed by T cell depletion and add-back experiments in congenic heterozygote and nude mice. Depletion and add-back experiments using NK cells would be a promising complimentary approach.

The roles of mediators other than type I IFN in the induction of salmonella resistance by MHV and in natural salmonella resistance needs to be determined. It is unlikely that type I IFN induction could account for all the effects on salmonella resistance caused by MHV infection. For example, we did not detect any type II IFN after MHV infection, but local effects cannot be ruled out. The best way to determine the role of gamma-IFN would be to use anti-gamma-IFN neutralizing monoclonal antibody to try to block natural resistance in naive mice and induction of salmonella resistance by MHV. Tumor necrosis factor (or lymphotoxin) also could have a role in resistance to salmonella; this could also be tested by blocking with specific monoclonal antibody. It is very possible that an interaction of other mediators with type I IFN could underlie induced resistance to salmonella.

A final, long-term goal of studies of this type is to develop methods for stimulating host defenses against salmonella and other intracellular pathogens. Although early interferon therapy did not live up to expectations, in the future it may be possible to use combinations of mediators to achieve the desired treatment effect without serious side effects.

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Major Subject Experimental Pathology

Title of Dissertation Effects of Mouse Hepatitis Virus Infection on the Resistance of Mice to Salmonella typhimurium

Dissertation Committee:

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