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Generation of congenic mouse strains by introducing the virus-resistant genes, Mx1 and Oas1b, of feral mouse-derived inbred strain MSM/Ms into the common strain C57BL/6J

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Abstract

Mx1 (Myxovirus resistance protein) and Oas1b (Oligoadenylate synthetase-1), induced by type 1 interferon (IFN), play a role in early antiviral innate immunity by inhibiting the replication of viruses. In mice, Mx1 and Oas1b confer resistance to the infection of orthomyxoviruses including influenza viruses and flaviviruses including West Nile viruses, respectively. Laboratory mice have been used to study the mechanisms of the pathogenesis of these virus infections; however, it is possible that they are not a suitable model system to study these viruses, since most of the inbred laboratory mouse strains lack both genes. It has been reported that feral mouse-derived inbred strains show resistance to the infection of these viruses due to the presence of intact both genes. In this study, we generated congenic strains in which the Mx or Oas locus of the MSM/Ms (MSM) mouse was introduced to the most widely used mouse strain, C57BL/6J (B6). B6.MSM-Mx mice showed resistance to the infection of influenza virus but not of West Nile virus. On the other hand, B6.MSM-Oas mice showed resistance to the infection of West Nile virus but not of influenza virus. Our results indicate that Mx1 and Oas1b show highly antiviral specificity in mice possessing the same genetic background. Therefore, these congenic mice are useful for not only infection study but also investigation of host defense mechanism to these viruses.

Key words: congenic mouse, flavivirus, Mx1, Oas1b, orthomyxovirus,
Introduction

Type 1 interferons (IFNs), IFN α/β, are produced and secreted from virus-infected cells and cause the surrounding cells to induce a number of cellular proteins, including the Mx (Myxovirus resistance protein) and Oas (Oligoadenylate synthetase) genes. These IFN-inducible proteins play important roles in the host's innate defense by inhibiting viral replication. Mx proteins can be found in a wide variety of organisms including mammals, birds, fish, and even invertebrate species and belong to the dynamin superfamily of high molecular weight GTPases. In mice, two Mx genes, Mx1 and Mx2, have been identified to locate in the Mx locus on Chromosome (Chr) 16. Mx1 has been identified as a gene encoding an anti-viral protein to orthomyxoviruses including influenza viruses. Oas family proteins are also highly conserved among many species. In virus-infected cells, Oas proteins are activated by the binding of viral double-stranded RNAs and are known to synthesize 2’-5’ oligoadenylate (2-5A). The 2-5A subsequently binds to and activates latent ribonuclease RNase L, resulting in the degradation of viral RNAs and the enhancement of IFN signaling. In mice, the Oas locus locates on Chr 5 and is composed of the Oas gene cluster, consisting of Oas1a-h, Oas2, and Oas3. Among these genes, Oas1b has been identified as a flavivirus-resistant gene.

Susceptibility to viruses has been believed to be associated with a genetic background in humans and other animals. This genetic effect is often complex and difficult to identify, since it is further modified by environmental factors. Laboratory mice such as B6 afford a useful alternative for the study of host defenses against infections, because variations among strains allow the identification of the genes associated with resistance or susceptibility to virus infection. However, as B6 mice lack the important Mx1 and Oas1b genes, they are not, perhaps, the most suitable model system for the study of both orthomyxo- and flaviviruses. Therefore, we established the congenic strains, B6.MSM-Mx and B6.MSM-Oas that carry the Mx and Oas locus, respectively, from the Japanese feral mouse-derived inbred strain, MSM/Ms. These congenic mice were found to be more resistant to lethal challenge with the two highly pathogenic viruses than were the original B6 mice. These congenic mice provide a useful model for the study of not only the antiviral function of Mx1 and Oas1b, but also the infectious mechanism of these viruses in humans and other animals.

Materials and Methods

Generation of congenic strains: The laboratory mouse strain, B6 was purchased from Charles River Japan (Tokyo, Japan) and the feral mouse-derived inbred strain MSM/Ms was provided by Prof. T. Shiroishi, National Institute of Genetics, Japan. Mx and Oas congenic mice were generated using the speed congenic method. Animal breeding rooms were maintained at 22 ± 4°C and 50 ± 20% relative humidity with a 12-hr light-dark cycle. Research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Veterinary Medicine, Hokkaido University. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University.
Genotyping was performed by polymerase chain reaction (PCR) with tail DNA. The forward and reverse primers for the Mx1 gene were designed based on the sequence of the MSM genome corresponding to the deleted-region of the B6 genome; GTGACCTTTGAACCTGCTTCTCT (intron 10) and GCAGACTCTTCCAGGGCTTTGA (exon 11) as described previously. PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. The forward and reverse primers for the Oas1b (accession number: NM_001083925) were, GCTCAAGGCGAGT CAGAC (nt 15–33 of exon 3) and TCAAAC TTCACCTCTCCAGC (nt 231–251 of exon 3), respectively. PCR products were digested with 2 U Hinfl for 1 hr, followed by electrophoresis in 8% acrylamide gels and visualization by ethidium bromide staining. Microsatellite markers used for genotyping are listed in Table 1.

Expression of intact Mx1 and Oas1b genes in congenic: Eight-week-old female B6, B6.

Table 1. List of microsatellite markers used for the marker-assisted speed congenic methods

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MSM-Mx, and B6.MSM-Oas mice were injected intraperitoneally with 200 μg of the synthetic double-stranded RNA (dsRNA) analog, poly (I:C) (GE Healthcare, UAS). Animals were sacrificed at 24 hr after the injection and the spleens were dissected. Total RNAs were isolated from the spleens using TRIzol reagent (Invitrogen, USA), and cDNAs were generated with oligo (dT) primers using ReverTra Ace (TOYOBO, Japan). PCR was performed using the following primers: ACGATGGATTCTGTGAATAATCTGT (nt 211–235) and TCTAGATGCCGTGTTAACTCG GAGAAATT (nt 2,095–2,121) for Mx1; GGCTG CAGAGGTATTAGCTGGACCT (nt 36–60) and CAGGAGTGCAATATCCAAAGACA (nt 1202–1226) for Oas1b; and TGATGGTGGGAA GGGTCAG (nt 207–226) and GAAAGCTGGAA GAGGCTG GAAAAGAGCCTC (nt 854–873) for mouse Actb (NM_007393). PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. Sequences of the Oas1b PCR products were determined with an ABI Prism 377 DNA Sequencer and an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

Experimental infection of mice with influenza virus and West Nile virus: To assess the lethality of viruses in the congenic mice, 8-week-old male mice were infected intranasally with 30 μl inoculum containing $10^5$–$10^7 \times 50\%$ of lethal dose (LD$_{50}$) of a highly pathogenic avian influenza strain, A/whooper swan/Mongolia/3/2005 (H5N1), diluted with phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin G and 100 μg streptomycin under anesthesia with 50 mg/kg sodium pentobarbital (Somnopentyl, Schering-Plough Animal Health U. S. A). The LD$_{50}$ value was calibrated using 8-week-old B6 mice. After infection, mice were monitored daily for 21 days. For West Nile virus infection, 6-week-old male mice were infected intraperitoneally with 200 μl inoculum containing 1 or 10 plaque forming units (PFUs) of West Nile virus 6LP strain diluted with PBS under anesthesia with diethyl ether inhalation. After infection, mice were followed up for 4 weeks.

Results

Generation of Mx1 and Oas1b congenic mice

Congenic strains were generated using a marker-assisted speed congenic strategy as reported previously[17]. To generate congenic mice in which the Mx or Oas locus from MSM mice was introduced into the genetic background of B6 mice, the B6 and MSM alleles of Mx1 and Oas1b were determined to select candidate mice. In the Mx1 locus of B6 mice, a large deletion from exon 9 to 11 was reported previously[2,33]. Therefore, primers were designed for amplifying the region from intron 10 to exon 11 to distinguish the B6 allele from the MSM allele as reported previously[2]. As shown in Fig. 1A, these primers can detect MSM allele. On the other hand, since a single nucleotide polymorphism has been found in the Hinfl site of the Oas1b gene in the MSM allele (Fig. 1B), genotyping was performed by digestion of the PCR products of the Oas1b gene with Hinfl (Fig. 1C). The ‘best’ male mice, those carrying the most homozygous B6 alleles in 134 microsatellite markers with heterozygosity in the Mx1 or Oas1b gene, were selected for breeding next generation. Backcrossing was performed six and seven times for exchanging to the B6 genetic background in the B6.MSM-Mx and B6.MSM-Oas mice, respectively. Finally, heterozygous sibling pairs were mated and homozygous mice were selected. To estimate the length of the chromosomal regions derived from MSM mouse, the genotype and position of microsatellite markers surrounding the Mx or Oas locus were confirmed (Fig. 2). As shown in Fig. 2A, the genotypes of D16Mit71 and D16Mit106 in the B6.MSM-Mx mice were homozygous MSM (M/M), whereas that of D16Mit20 was homozygous B6 (B/B), suggesting that the region between D16Mit71 and D16Mit106 was derived from MSM and recombination occurred at two points between D16Mit20
Fig. 1. Detection of the B6 and MSM alleles of the Mx1 and Oas1b genes. (A) The results of PCR amplification of the Mx1 gene. The alleles of MSM but not of B6 mice show the PCR product. (B) Schematic diagram of the Oas1b gene in B6 and MSM mice. The arrows show PCR primers used for Oas1b genotyping. The lower diagram shows the expected results of Hinfl digestion after PCR amplification. H; Hinfl site. (C) The result of genotyping of the Oas1b gene. The 28-bp and 142-bp bands are derived from the MSM allele.

Fig. 2. Schematic diagrams of the genomic structure surrounding Mx1 and Oas1b genes in the B6.MSM-Mx (A) and B6.MSM-Oas (B) congenic strains, respectively. Black and gray bars represent the MSM-derived and B6-derived genomes, respectively. Dotted bars represent recombined regions between the MSM and B6 genomes. The numbers to the left of the bars represent physical locations based on the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview).
Congenic mouse strains introduced \textit{Mx1} and \textit{Oas1} genes

and \textit{D16Mit71}, and between \textit{D16Mit106} and telomere. On the other hand, the genotypes of \textit{D5Mit24}, \textit{D5Mit367}, and \textit{D5Mit242} in B6. MSM-\textit{Oas} mice were M/M, whereas those of \textit{D5Mit109} and \textit{D5Mit168} were B/B (Fig. 2B), suggesting that the region between \textit{D5Mit24} and \textit{D5Mit242} was derived from MSM mice and recombination occurred at two points between \textit{D5Mit109} and \textit{D5Mit24}, and between \textit{D5Mit242} and \textit{D5Mit168}.

Next we confirmed by RT-PCR that \textit{Mx} or \textit{Oas1b} mRNA was transcribed from both MSM alleles in congenic mice. After induction of IFN using poly (I:C) injection, expression of the \textit{Mx1} gene in the spleens of B6.MSM-\textit{Mx}, MSM, and B6 mice was analyzed. Intact \textit{Mx1} could be distinguished from the mutant type due to the large deletion in the \textit{Mx1} gene of the B6 mouse (Fig. 3A). In addition, no \textit{Mx2} gene expression was detected in the spleens of B6.MSM-\textit{Mx} and MSM mice (Fig. 3A). On the other hand, since \textit{Oas1b} genes expressed from the B6 and MSM mice could not be distinguished by the length of RT-PCR products (Fig. 3B), we determined the origin of the \textit{Oas1b} gene by DNA sequencing. A nonsense mutation was observed in the B6 \textit{Oas1b} cDNA, whereas the sequence of the \textit{Oas1b} cDNA in the B6.MSM-\textit{Oas} mice was identical to that of MSM mice (data not shown). Interestingly, both genes are basally expressed in B6 background and are more induced by dsRNA stimulation than those of original MSM mice (Fig. 3). This result is in agreement with previous report showing that MSM strain is hyporesponsive to poly (I:C) due to a mutation in toll-like receptor 3 activated by dsRNA\textsuperscript{35}. Thus, we have confirmed that these congenic strains are able to express the intact \textit{Mx1} and \textit{Oas1b} genes by the stimulation of dsRNA.

\textbf{Experimental infection of congenic strain with orthomyxovirus and flavivirus}

We performed viral injection to confirm whether these congenic mice were resistant to the infection of orthomyxovirus and flavivirus. A highly pathogenic avian influenza virus and West Nile virus were selected as the representative orthomyxo- and flaviviruses, respectively. When

![Fig. 3](image_url)

\textit{Fig. 3. Expression of intact }\textit{Mx1} \textit{and }\textit{Oas1b} \textit{genes in congenic mice.} (A) Expression of \textit{Mx1} and \textit{Mx2} genes in the spleen tissues of B6, MSM, and B6.MSM-\textit{Mx} mice in response to poly (I)/(C). (B) Expression of \textit{Oas1b} gene in the spleen tissues of B6, MSM, and B6.MSM-\textit{Oas} mice in response to poly (I)/(C).
infected with a $10^2 \text{LD}_{50}$ dose of influenza A virus, all B6 and B6.MSM-Oas mice died within 14 days after infection, whereas 5 of 7 (71%) B6.MSM-Mx mice survived. When B6.MSM-Mx mice were infected with 2 higher doses, $10^3 \text{LD}_{50}$ and $10^4 \text{LD}_{50}$, 6 of 7 (86%) and 7 of 7 (100%) B6.MSM-Mx mice survived, respectively (Fig. 4A). On the other hand, all B6.MSM-Oas mice infected with 1 and 10 PFUs of West Nile virus survived, whereas most of B6 and B6.MSM-Mx mice died within 14 days after infection. The survival rate in each experiment was as follows; B6 mice infected with 1 PFU, B6 with 10 PFU, B6.MSM-Mx with 1 PFU, and B6.MSM-Mx with 10 PFU were 20% (3 out of 15), 7% (1 out of 14), 10% (1 out of 10), and 30% (3 out of 10), respectively (Fig. 4B). These results indicate that Mx1 and Oas1b have specific anti-virus activity against influenza- and West Nile viruses, respectively, in mice with the same B6 genetic background.

**Discussion**

Orthomyxovirus and flavivirus are considered to be important viruses from both the medical and sanitary position. Influenza A, B, and C viruses, classified as orthomyxoviruses, cause the epidemic respiratory disease known as ‘flu’ in humans that spreads worldwide. Last century, pandemic influenza emerged several times. Further, a new emerging pandemic influenza virus is much concerned\textsuperscript{13,15,21}. On the other hand, 20–30 members of the flavivirus family are known to be involved in human diseases. Some of these viruses cause severe diseases such as fatal encephalitis and hemorrhagic fevers in humans\textsuperscript{10,31}. Last century, yellow fever virus, Dengue virus, Japanese encephalitis virus, tick-borne encephalitis virus, and West Nile virus caused large outbreaks worldwide\textsuperscript{10}. Taken together, infectious diseases associated with orthomyxoviruses and flaviviruses are the subject of much important study. In mice, both Mx1 and Mx2 proteins have been identified, and Mx1 localizes in the nucleus and inhibits virus polymerase activity\textsuperscript{5,14,22,25,39}. Therefore, mice carrying intact Mx1 show resistance to orthomyxoviruses such as influenzavirus\textsuperscript{1,33}. B6.MSM-Mx and B6 mice showed notably different survival rates after a challenge with a highly pathogenic avian influenza virus. Although it remains to be determined whether Mx in all vertebrates possesses anti-viral
activity against orthomyxoviruses, anti-viral activity of the Mx protein has been shown in rodents, human, and other animals, suggesting that laboratory mice lacking these genes may not reflect the normal infectious conditions in humans and animals. In addition, these data indicate that studies performed using mice lacking Mx would lead to incorrect evaluations of viral virulence, effects of vaccine, drug and therapy and so on. MSM mice do not express Mx2, and B6.MSM-Mx mice do not recover Mx2 expression, suggesting that the absence of Mx2 expression in MSM mice is not due to the MSM genetic background but due to the presence of a putative cis-acting element in the Mx locus. Mouse Mx2 protein localizes in the cytoplasm in the same manner as the Mx proteins in humans and some animals. Mouse Mx2 can inhibit the replication of negative-stranded RNA viruses that replicate in the cytoplasm such as vesicular stomatitis virus and hanta virus, but not those that replicate in the nucleus such as influenza virus. On the other hand, Mx proteins in humans and some animals localize in cytoplasm and can inhibit influenza virus replication. The reason for the differential anti-viral activity between mouse Mx2 and Mx proteins of other species is unknown.

Oas genes are induced by type 1 IFN, and synthesize 2′-5′A, and consequently RNA degradation, by activating latent RNase L. In mice, a gene cluster has been identified on Chr 5 that is comprised of ten Oas family genes. Although mouse Oas1b is one of these genes, Oas1b protein is not regarded as a typical Oas protein, because it lacks enzymatic activity. Although mice carrying intact Oas1b show resistant in flavivirus infection, the mechanism by which Oas1b confers resistance to the infection of flaviviruses on mice remains unclear. In recent reports, the Oas1b gene of wild-derived mice was shown to confer differential resistance to the infection of flaviviruses due to the polymorphisms. In our study, B6.MSM-Mx but not B6.MSM-Oas mice showed resistance to the infection of influenza virus. On the other hand, B6.MSM-Oas but not B6.MSM-Mx mice showed resistance to the infection of West Nile virus, suggesting that the Oas1b protein of MSM mice possesses anti-West Nile virus characteristics but murine Mx1 protein does not. In addition, these results indicate that Mx1 and Oas1b specifically inhibit influenza virus and West Nile virus replications, respectively.

As there is only limited information on the mechanisms of the pathogenesis of virus infection and the role of host innate immune response in humans, animal models are necessary to identify relationship between virus and host defense in infectious diseases. Mice are frequently used as an animal model to study the viral virulence and vaccine efficacy before using other larger animals. However, standard laboratory mice do not possess certain key components of the innate immune system that mediates protection against the infection of these viruses. It has been reported that the proinflammatory cytokines, such as interleukin 1, interleukin 6, and macrophage inflammatory protein-1, are significantly increased in highly pathogenic influenza-infected mice; however, the course of the disease and the extent of virus replication and spread in these knockout mice were not different from those observed in wild-type mice. On the other hand, IFN α/β receptor-deficient mice are highly susceptible to pathogenic influenza virus. These results suggest a role for IFN signaling, including Mx1, which is essential for protection of the host in the early stages of infection in mice. Thus, the congenic mice generated in this study are useful for the further investigation of orthomyxovirus and flavivirus infectious diseases, particularly, the precise mechanism and timing of the interplay between components of pro- and anti-inflammatory signaling pathways, and may allow the eventual identification of an effective target on these viruses.
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