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NOTES

Tick-Borne Thogoto Virus Infection in Mice Is Inhibited by the Orthomyxovirus Resistance Gene Product Mx1

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We show that tick-transmitted Thogoto virus is sensitive to interferon-induced nuclear Mx1 protein, which is known for its specific antiviral action against orthomyxoviruses. Influenza virus-susceptible BALB/c mice (lacking a functional *Mx1* gene) developed severe disease symptoms and died within days after intracerebral or intraperitoneal infection with a lethal challenge dose of Thogoto virus. In contrast, *Mx1*-positive congenic, influenza virus-resistant BALB.A2G-*Mx1* mice remained healthy and survived. Likewise, A2G, congenic B6.A2G-*Mx1* and CBA.T9-*Mx1* mice (derived from influenza virus-resistant wild mice) as well as *Mx1*-transgenic 979 mice proved to be resistant. Peritoneal macrophages and interferon-treated embryo cells from resistant mice exhibited the same resistance phenotype in vitro. Moreover, stable lines of transfected mouse 3T3 cells that constitutively express Mx1 protein showed increased resistance to Thogoto virus infection. We conclude that an Mx1-sensitive step has been conserved during evolution of orthomyxoviruses and suggest that the *Mx1* gene in rodents may serve to combat infections by influenza virus-like arboviruses.

Humans have evolved a mechanism of controlling influenza virus infections through the effect of interferon (IFN)-induced cytoplasmic MxA protein (28, 29). A similar protein, Mx1, is produced by mice but is located in the nucleus (6, 18, 34). Specific resistance of laboratory and wild mice (12) to several strains of orthomyxoviruses is determined by alleles at the IFN-regulated *Mx1* locus on chromosome 16 (reviewed in references 10 and 33). The Mx1 protein has intrinsic antiviral activity against orthomyxoviruses and acts by blocking primary transcription of the viral RNA genome (21, 28), a process that takes place in the nuclei of infected cells. The reason why mice have evolved a system that specifically inhibits orthomyxovirus infections is unclear given that mice are not natural hosts of influenza viruses (12). However, two tick-borne members of the *Orthomyxoviridae* family, Thogoto (THO) and Dhori viruses, have been recognized (1, 4, 8, 24, 26, 27), and these are likely to encounter small rodents in their natural environment (5). In this study, we investigate the effect of Mx1 protein on THO virus infection.

Resistance of *Mx1*-positive mice to lethal THO virus infection. THO virus is known to cause acute liver failure and death in outbred albino mice when given intraperitoneally (i.p.) (7). However, influenza virus-resistant *Mx1*-positive mice have not previously been investigated. We therefore examined the response of *Mx1*-positive mice to infection with THO virus. Stock virus of the Sicilian (SiAr 126) isolate of THO virus (1) was prepared by three serial liver passages in suckling BALB/c mice. It contained 3×10^6 PFU, corresponding to 10^6 i.p. 50% lethal doses per ml as determined in 3-month-old male BALB/c mice. In a first experiment, influenza virus-resistant A2G as well as *Mx1*-positive congenic BALB/c and C57BL/6 mice (16) were infected i.p. with a lethal dose of THO virus and compared with similarly infected influenza virus-susceptible BALB/c and C57BL/6 mice. Mice of both *Mx1*-negative strains became progressively ill and succumbed within 5 to 8 days to an acute degenerative hepatitis characteristic of THO virus infection (7). In contrast, all *Mx1*-positive mice survived without any symptoms (Table 1). Surviving animals developed specific antibodies as assessed in paired serum samples indicating seroconversion due to asymptomatic infection (data not shown). In a second experiment, THO virus was given intracerebrally. In addition, CBA.T9-*Mx1* mice, which carry the $Mx1^+$ allele of a wild mouse, were included. The wild mouse allele had originally been introduced into the CBA/J genetic background by repeated backcrossings from an influenza virus-resistant Lake Casitas *Mus musculus domesticus* mouse called T9 (11, 12). Again, influenza virus-susceptible CBA/J mice uniformly died within 7 days, whereas all influenza virus-resistant animals survived, including the wild mouse-derived strain (Table 1). Likewise, *Mx1*-positive mice resisted a subcutaneous THO virus challenge that killed five of seven Mx1-negative mice (Table 1). Seroconversion occurred in all survivors, indicating successful take of the challenge virus (not shown). These data demonstrate that THO virus resistance is expressed systemically in many organs and encompasses various routes of infection, including the subcutaneous route, which probably mimics best the natural mode of THO virus transmission. Thus, only mice carrying a functional *Mx1* allele resist infection with THO virus as described for influenza A virus.

THO virus growth in livers of susceptible and resistant mice. We analyzed THO virus growth in the liver in more detail. THO virus grew to high titers in adult BALB/c mice, reaching $10^{7.5}$ 50% tissue culture infective doses per g of liver homogenate on day 4 when most animals were moribund. In congenic BALB.A2G-*Mx1* mice, no virus growth could be demonstrated (Fig. 1). Virus titers remained below the detection limit of $10^{2.5}$ 50% tissue culture infective doses at each time point analyzed. This result indicated that THO virus ei-

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TABLE 1. Genetic resistance of *Mx1*-positive mice to THO virus infection

Expt	Mouse		Route of infection[a]	Mortality[b]
	Strain	*Mx1* genotype		
1	BALB/c	−	i.p.	14/16
	C57BL/6	−	i.p.	7/7
	BALB.A2G-*Mx1*	+	i.p.	0/13
	B6.A2G-*Mx1*	+	i.p.	0/5
	A2G	+	i.p.	0/6
2	CBA/J	−	i.c.	11/11
	CBA.T9-*Mx1*	+	i.c.	0/4
	A2G	+	i.c.	0/4
3	C57BL/6	−	s.c.	5/7
	BALB.A2G-*Mx1*	+	s.c.	0/4
	A2G	+	s.c.	0/3
4	C57BL/6	−	i.p.	9/10
	C3H	−	i.p.	5/5
	979	Transgenic	i.p.	0/10
	A2G	+	i.p.	0/10

[a] Mice (6 to 18 weeks old) of either sex were infected with challenge doses of 3×10^5 PFU for i.p., 10^3 PFU for intracerebral (i.c.), and 2×10^3 PFU for subcutaneous (s.c.) infection. In experiment 4, mice were infected i.p. with 10^3 PFU. Five animals of strains 979, A2G, and C57BL/6 were pretreated with 100 μg of the IFN inducer poly(I)-poly(C) 6 h before infection (3). The surviving C57BL/6 mouse belonged to the group not pretreated with poly(I)-poly(C).

[b] Number of dead animals on day 15/total number of mice infected.

ther did not reach the liver of resistant animals or did not replicate there to a discernible degree.

The livers of susceptible *Mx1*-negative mice became pale yellow, swollen, and friable 72 to 96 h after THO virus infection. Histologically, lesions consisted of focal to widespread liver cell necrosis with almost no inflammatory cell infiltrates (Fig. 2A). Cellular degeneration was characterized by acidophilic hyalinization of the cytoplasm, pyknotic, or karyorrhectic nuclei and the formation of typical Councilman bodies. Necrosis progressed until little recognizable liver structure remained. In sharp contrast, infected *Mx1*-negative livers remained macroscopically normal. Hematoxylin-and-eosin-stained sections

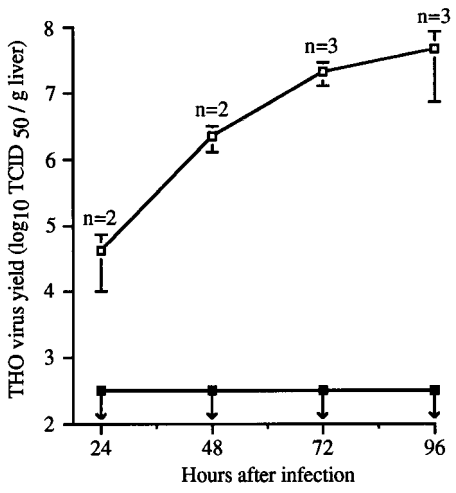

FIG. 1. Virus growth in livers of susceptible and resistant THO mice. Adult BALB/c (□) and BALB.A2G-*Mx1* (■) mice were infected i.p. with 10^3 PFU of THO virus. Livers were removed at the times indicated and assayed for infectivity. Each point represents the mean virus titer ± standard error of the mean for the number of mice indicated. $TCID_{50}$, 50% tissue culture infective dose.

exhibited no histological lesions either at 96 h after infection (Fig. 2B) or at later times (not shown).

To provide evidence that resistant animals survived because Mx1 protein was induced by the infecting virus, we attempted to detect Mx1 in vivo. Frozen cryostat sections of livers from infected mice were processed by double indirect immunofluorescence using specific antibodies to reveal simultaneously the presence of THO viral antigens and Mx1 protein as described previously (2). To visualize Mx1 protein, the antipeptide antibody AP5 (23) was used. To label THO antigens, hyperimmunized guinea pig antisera (20) were used. As expected, resistant animals infected 72 h earlier with 10^3 PFU of THO virus showed intense staining for Mx1 protein in most liver cells but no THO viral antigens (Fig. 2D and F). It was difficult to find viral antigen-expressing cells in the livers of resistant animals at all stages of infection investigated. Exceptionally, hepatocytes exhibited the characteristic staining pattern of THO virus-infected cells (not shown), indicating that infection was restricted to rare and tiny lesions. Liver sections of susceptible *Mx1*-negative mice showed many THO viral antigen-producing cells at 72 h after infection but no Mx1 protein (Fig. 2E and C). The number of viral antigen-positive cells increased with time, demonstrating virus spread throughout the infected organ (not shown).

Macrophages express resistance phenotype. A peculiarity of orthomyxovirus-resistant mice is the capacity of their macrophages to express virus resistance in vitro (15, 22). This phenomenon is due to high Mx1 protein levels induced in macrophages by endogenous IFN acting in vivo (6, 13). We therefore anticipated that freshly explanted *Mx1*-positive macrophages would not be permissive for THO virus multiplication. Peritoneal macrophages were obtained from 6- to 8-week-old mice after stimulation by i.p. injections of 2 ml of 0.3% fluid thioglycolate medium (Difco Laboratories, Detroit, Mich.) and were cultured as described previously (22). THO (SiAr 126) virus stocks grown in BHK-21 cells containing 8.3×10^7 PFU/ml (as determined in Vero cells) were used for all tissue culture experiments. Peritoneal macrophages from B6.A2G-*Mx1* mice remained unaffected by a large challenge dose of THO virus, whereas *Mx1*-negative macrophages from C57BL/6 mice showed a pronounced cytopathic effect with rounding of cells and detachment from the culture dish after 96 h of infection (not shown). Pretreatment of susceptible macrophages with exogenous IFN type I did not prevent the cytopathic effect and decreased virus production approximately 10-fold, indicating that IFN action against THO virus was inefficient in the absence of a functional $Mx1^+$ gene. Resistant macrophages did not produce detectable infectious progeny virus irrespective of IFN treatment (not shown).

IFN inhibits THO virus multiplication in *Mx1*-positive but not *Mx1*-negative embryo cells. Parallel cultures of congenic *Mx1*-positive and *Mx1*-negative mouse embryo cells (35) were treated for 15 h with recombinant human IFN-αB/D, (a gift from Ciba Geigy Ltd., Basel, Switzerland), known to be active on mouse cells (17). Cells were then infected at a multiplicity of 0.1 with either THO virus or influenza A virus strain FPV-B (19). Untreated control cultures were infected in the same way. Virus yields were determined at various time points thereafter. Figure 3 shows that IFN markedly inhibited the multiplication of both viruses in *Mx1*-positive embryo cells but exerted only a slight inhibition or delay of viral multiplication in *Mx1*-negative cultures. These findings correspond well to the in vivo results shown above and demonstrate again that THO virus behaves like influenza virus in its sensitivity to the Mx1 antiviral mechanism activated by IFN.

Mx1 protein mediates resistance to THO virus infection. To

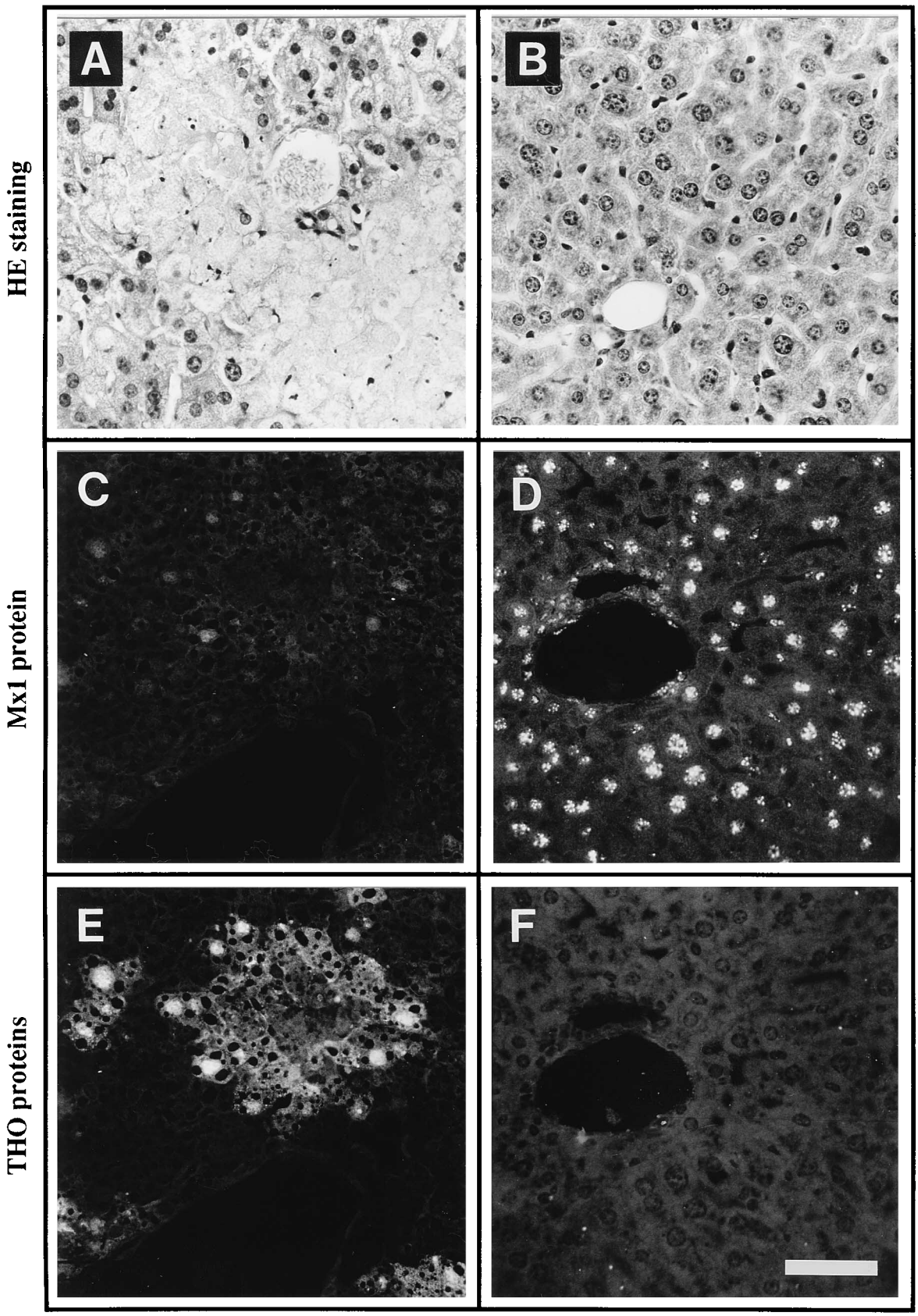
BALB/c
BALB.A2G-*Mx1*
HE staining
Mx1 protein
THO proteins
A
B
C
D
E
F

FIG. 2. Histology and double indirect immunofluorescence of liver sections of susceptible and resistant mice infected with THO virus. Mice were infected i.p. with 10^3 PFU of THO virus. Hematoxylin-and-eosin (HE)-stained sections of BALB/c (A) and BALB.A2G-*Mx1* (B) mice were prepared 96 h after infection. Cryostat sections of BALB/c (C and E) and BALB.A2G-*Mx1* (D and F) mice were prepared 72 h after infection. They were simultaneously stained for Mx1 protein (C and D) and THO viral proteins (E and F) by using specific antibodies. Bar = 50 μm.

exclude the remote possibility that THO virus resistance of *Mx1*-positive mice (and of cells obtained from these animals) was due to a distinct IFN-regulated gene closely linked to the *Mx1* locus on chromosome 16, *Mx1*-transgenic mice were analyzed. We infected 10 mice of the high-responder line 979, known to exhibit the influenza virus resistance phenotype in vivo (3), with a lethal challenge dose of THO virus and also tested appropriate controls. Since the *Mx1* cDNA is linked to the IFN-inducible *Mx1* promoter in these mice, half of the experimental animals were pretreated with the IFN inducer poly(I)-poly(C) before infection in order to enhance transgene expression (3). All *Mx1*-transgenic mice survived lethal THO virus infection irrespective of pretreatment (Table 1). Likewise, all 10 *Mx1*-positive control A2G mice survived, whereas the C57BL/6 and C3H mice representing the *Mx1*-negative genetic background of the transgenic line succumbed with one exception. As expected, poly(I)-poly(C) treatment was without noticeable effect in *Mx1*-negative mice. These results indicated that Mx1 protein, and not some other factors, was mediating THO virus resistance in vivo.

To further substantiate that the Mx1 protein was responsible for THO virus inhibition, we also investigated permanently transfected 3T3 cells known to be resistant to influenza virus infection because they express high levels of Mx1 protein in a constitutive manner (28, 34). As expected, these cells exhibited a high degree of resistance to THO virus: in five independent experiments, the Mx1 protein performed the same protection against both THO virus and influenza virus (average protection values of 99.05% ± 0.46% and 98.79% ± 0.81%, respectively), whereas growth of the rhabdovirus vesicular stomatitis virus was unaffected. A representative experiment is shown in Fig. 4. Again, these results indicate that Mx1 protein is the mediator of resistance to THO virus.

A priori, THO virus sensitivity to the Mx1 antiviral mechanism was improbable for several reasons. Unlike influenza virus, THO virus is an arbovirus able to replicate in invertebrate cells and is transmitted by tick bite. Its genome consists of only six RNA segments (36), whereas influenza A and B viruses have eight segments. To date, two genomic segments have been cloned and sequenced; one (segment 3) encodes a PA-equivalent protein which is considerably smaller than those of influenza viruses and has only 11 to 12% amino acid identity (36). THO segment 4 encodes a glycoprotein that is unrelated to any influenza virus glycoproteins but homologous to gp64 of baculoviruses (25). Furthermore, preliminary evidence suggests that transcription initiation of THO virus differs from that found for influenza virus because it does not involve the stealing of 10 to 12 nucleotides from cellular mRNA (27). Nevertheless, we now find that influenza virus and THO virus both exhibit an Mx1-sensitive step(s) in their multiplication cycles, indicating a close evolutionary relationship between these otherwise distantly related viruses. The THO virus prototype strain from Kenya (designated THO IIa) was indistinguishable in this respect from the Sicilian isolate (SiAr 126) described here (7a). The present results strongly support the classification of THO virus as a new member of the *Orthomyxoviridae*, as recently proposed (27). Interestingly, similar results were obtained with Dhori virus (37), another tick-borne virus related to influenza viruses (27). Moreover, the nuclear rat Mx1 protein, which is closely related to mouse Mx1 protein (23), was also able to block THO virus multiplication (30).

In the case of influenza A virus, mouse Mx1 protein has been shown to inhibit the accumulation of primary transcripts that are synthesized by the incoming virion polymerase in the nuclei of infected cells (21, 28). It seems reasonable to assume that Mx1 protein, being in the nucleus, blocks the same event in THO virus-infected cells. Further insight into the molecular biology of THO virus should help to unravel the molecular target structures recognized by Mx1.

The *Mx1* gene is regulated tightly and exclusively by IFN type I (34, 35). It is usually quiescent in cells of uninfected animals but is inducible to high levels by virus infection (33). Clearly, THO virus infection of *Mx1*-positive mice induced massive Mx1 protein expression in the liver and probably most

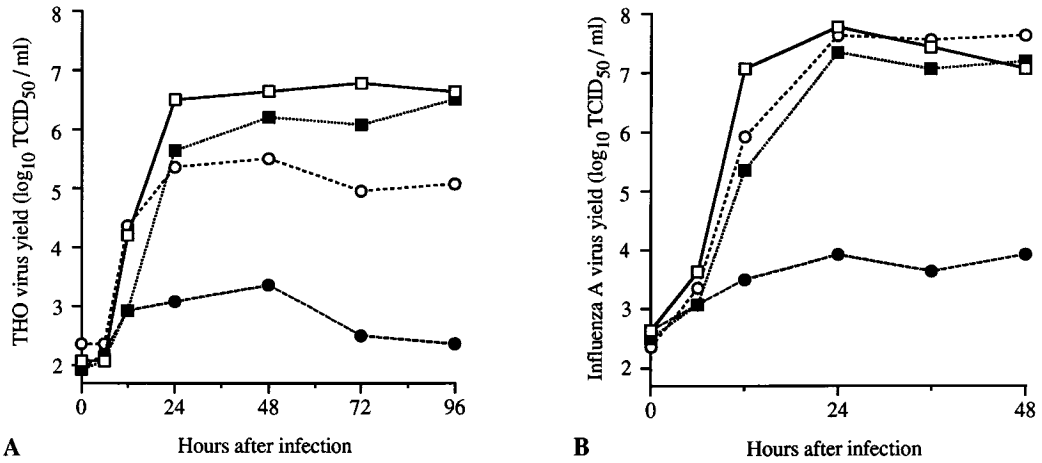

FIG. 3. IFN-treated *Mx1*-positive embryo cells are resistant to THO virus infection. Mouse embryo cells prepared from BALB/c (□, ■) and BALB.A2G-*Mx1* (○, ●) mice were treated with 10^3 IU of IFN type I per ml for 15 h (■, ●) or were left untreated (□, ○). Thereafter, cells were infected with 0.1 PFU of THO virus (A) or influenza A virus strain FPV-B (B) per cell. Supernatants were assayed for infectivity at the times indicated. $TCID_{50}$, 50% tissue culture infective dose.

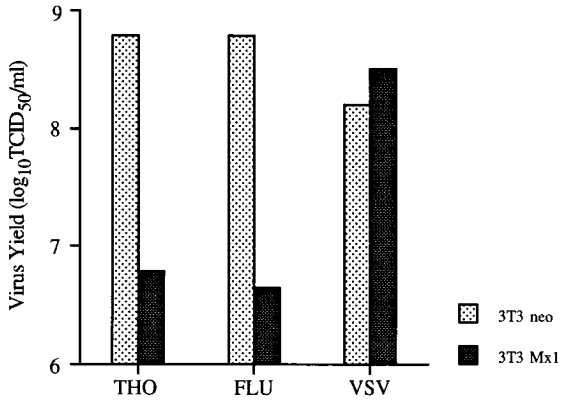

FIG. 4. Inhibition of THO virus multiplication by Mx1 protein. 3T3-mMx1 cells expressing murine Mx1 protein and control 3T3-SV2neo cells were infected with 0.1 PFU of THO virus (SiAr 126), influenza A virus (FLU; strain FPV-B), or vesicular stomatitis virus (VSV; serotype Indiana) per cell. Supernatants were assayed for infectivity at the times of maximal titers for each virus (12 h for vesicular stomatitis virus, 24 h for influenza virus, and 48 h for THO virus). $TCID_{50}$, 50% tissue culture infective dose.

other organs, indicating considerable virus multiplication and IFN production in vivo. Although the liver is known to be a major target organ (7) and was severely affected in *Mx1*-negative mice, virus growth in *Mx1*-positive livers was hardly detectable. Most likely, initial virus replication takes place outside the liver and most hepatocytes are antivirally protected by the time infectious virus reaches the organ. This is in contrast to the situation with a liver-adapted influenza A virus, TURH virus (9). This virus reaches the liver within a few hours before the Mx1 defense mechanism is in place. TURH virus then multiplies and forms localized liver lesions (14). These are self-limiting effects because of local IFN production and Mx1 expression in neighboring cells which become protected and restrict further viral spread (14, 33). A decisive difference between TURH and THO viruses is probably the slower growth rate of THO virus in mammalian cells (31). Hence, the Mx1 system gains time to respond, a phenomenon also seen in tissue culture. Contrary to TURH virus-infected animals, resistant animals infected with THO virus never showed any disease symptoms, even at early times of infection. In addition to the liver, other organs like the lung and brain escaped overt infection, whereas infectious virus could easily be isolated from these organs in susceptible *Mx1*-negative mice (7). It is conceivable that resistant macrophage populations play a crucial role in vivo. However, the site of initial virus multiplication has not been determined.

Finally, the present results may contribute to a better understanding of the biological function of the mouse Mx1 system. While influenza A viruses have been isolated from a variety of animal species, including pigs, horses, seals, and birds (38), no murine isolates exist and rodents are not considered to be natural hosts for influenza viruses. Why, then, should mice and rats possess a powerful defense mechanism against influenza A and B viruses? As shown here, $Mx1^+$ alleles found in laboratory and wild mice efficiently protect these animals from fatal infection with influenza virus-like arboviruses. It is tempting to speculate that $Mx1^+$ alleles serve a similar function in the wild. Under natural conditions, it is unknown whether wild-living mice become infected with THO virus. However, antibodies to THO virus have been detected in rats (5), and it is conceivable that infected ticks transmit the virus also to wild mice. Moreover, hitherto undetected arthropod-borne influenza viruses that are able to cause sporadic disease in wild-living mouse populations may exist.

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