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The M2 Gene Segment Is Involved in the Capacity of Reovirus Type 3 Abney to Induce the Oily Fur Syndrome in Neonatal Mice, a S1 Gene Segment-Associated Phenotype

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Oral inoculation of reovirus type 3 Abney (T3A) into neonatal mice induces hepatitis and the biliary atresia-associated oily fur syndrome (OFS), a phenotype previously linked to the S1 gene. We found that following oral inoculation, none of three T3A mutants, JH2, JH3, and JH4, containing different single amino acid substitutions in the M2 gene, induced the OFS or extensive liver necrosis. Similarly, reassortant viruses containing both a JH4-S1 and a JH4-M2 gene segment did not induce the OFS, whereas another reassortant containing a JH4-S1 gene and a M2 gene from reovirus type 3 Dearing fully recovered this capacity. Together, these results constitute the first evidence for the involvement of the M2 gene in the S1 gene-associated capacity of T3A to induce hepatobiliary disease in neonatal mice.

INTRODUCTION

Hepatitis and extrahepatic biliary atresia are common neonatal liver diseases in human (Sokol and Mack, 2001). Because infectious agents, such as members of the family *Reoviridae* (reoviruses, rotaviruses), could participate in the etiology of these liver pathologies, experimental models have been developed. Thus, almost 4 decades ago, hepato-encephalomyelitis in mice infected with reovirus type 3 was first reported (Stanley, 1961). More recently, hepatocellular necrosis and the biliary atresia-associated oily fur syndrome (OFS) were described in reovirus type 3 Abney (T3A)-infected mice inoculated by a natural route of infection for enteric viruses, the oral route (Wilson *et al.,* 1994). Genetic analysis, using reassortant viruses containing different combinations of RNA segments derived from T3A and from another reovirus strain, type 1 Lang (T1L), demonstrated an association between the OFS and the T3A S1 gene segment that encodes for the cell attachment protein σ1 (Wilson *et al.,* 1994). In mice, the S1 gene segment has been associated with the relative capacity of different reovirus strains to spread to the brain through nerves (Tyler *et al.,* 1986). Furthermore, this viral gene, together with the M2 gene segment that encodes for another major component of the reovirus outer capsid, μ1/μ1C, determines the relative capacity of reovirus strains to induce cell death by apoptosis (Tyler *et al.,* 1996; Barton *et al.,* 2001). In mice, the M2 gene has been associated with virulence (Rubin and Fields, 1980; Hrdy *et al.,* 1982) and with the relative capacity of different reovirus strains to generate suppressor T cells (Rubin *et al.,* 1981) and specific immunoglobulins (Mayor and Cuff, 1996) after oral inoculation. Thus, both σ1 and μ1/μ1C proteins play different critical roles in infection. In this study, we used T3A mutants and reassortant viruses to demonstrate that the M2 gene segment contributes to the capacity of reovirus T3A to induce the OFS, a phenotype genetically linked to the S1 gene segment.

RESULTS

Reovirus T3A induces an OFS and severe pathological changes in hepatic tissue when inoculated po into neonatal mice (Wilson *et al.,* 1994). Furthermore, mice eventually die of infection (Wilson *et al.,* 1994) and encephalitis may be the cause of death, as observed in mice infected with other neurotropic type 3 strains (Morrison *et al.,* 1991). Both capacity to induce the OFS and capacity to invade the brain through nerves are linked to the type 3 S1 gene segment (Tyler *et al.,* 1986; Wilson *et al.,* 1994). To uncover a potential role of the M2 gene segment in the capacity of T3A to induce these phenotypes, we inoculated groups of 1- to 2-day-old mice perorally (po) with increasing doses of T3A or with T3A mutant viruses (JH2, JH3, and JH4) bearing different single point mutations within the central portion of the M2 gene (Hooper and Fields, 1996). Mice were observed daily for oily fur development and lethality. A clear and severe OFS was observed in mice inoculated with a dose of T3A

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TABLE 1

T3 Abney Mutants of the M2 Gene Exhibit Altered Phenotypes Compared to T3A When Inoculated Perorally Into Neonatal Mice

Virus	Doses po (log_{10} PFU)[a]	Oily fur syndrome[b] (day of appearance)[c]	Mean day of death[d]	LD_{50}[e]
T3A	0 ($n = 10$)	+(10 ± 2)	17 ± 4	
	1.00–1.70 ($n = 7$–8)	+(9 ± 1)	16 ± 3	
	2.04–2.68 ($n = 6$–9)	+(8 ± 1)	13 ± 5	
	3.07–3.83 ($n = 7$)	+(8 ± 2)	13 ± 4	
	4.98 ($n = 7$–8)	+(8 ± 1)	13 ± 6	
	5.92 ($n = 10$)	+(5 ± 3)	13 ± 4	2
JH2	2.11 ($n = 10$)	−	−	
	2.90–2.93 ($n = 8$)	−	−	
	3.65–3.96 ($n = 8$)	−	−	
	4.84–4.87 ($n = 7$–8)	−	15 ± 0	
	5.84–5.90 ($n = 8$–9)	+(9 ± 1)	−	6.90
JH3	0.77 ($n = 8$)	−	−	
	1.90–2.14 ($n = 7$–8)	−	18 ± 5	
	3.14–3.87 ($n = 8$)	−	18	
	4.00–4.17 ($n = 8$)	−	14 ± 4	
	5.07–5.17 ($n = 7$–8)	−	12 ± 4	
	6.20 ($n = 8$)	−	12 ± 2	3.56
JH4	1.00 and 1.80 ($n = 7$–8)	−	−	
	2.39–2.90 ($n = 8$)	−	−	
	3.95–4.34 ($n = 8$)	−	−	
	5.00–5.17 ($n = 6$–7)	−	−	
	6.38 ($n = 9$)	−	−	
	7.14 ($n = 5$)	−	−	>7

[a] Doses of virus (log_{10} PFU) inoculated as determined in duplicates by standard plaque assay.

[b] Intensity of oily fur syndrome observed. The oily fur phenotype was scored as follows: (−) absence of oily fur, (+) severe oily fur covering the entire mouse and affecting the entire group of mice, including the mother. Data are from two representative independent experiments out of 3 (JH3), 5 (JH2 and JH4), or 6 (T3A) independent experiments performed.

[c] Day after inoculation at which the first oily fur patches appeared.

[d] Mean day of death after inoculation plus standard deviation.

[e] Number of PFU (log_{10}) resulting in 50% mortality following po inoculation of 1- to 2-day-old Swiss Webster mice calculated by the method of Reed and Muench.

as low as 1 PFU (Table 1). Furthermore, the intensity of the OFS in mice challenged with 1 or with 10^6 PFU was the same (Table 1). T3A-infected mice developed this syndrome as soon as 5 days postchallenge when inoculated with the highest dose of virus tested (10^6 PFU) and no later than 10 days after inoculation of the lowest dose (1 PFU). After symptom onset, animals died within 4 to 7 days. The calculated dose of virus required to produce 50% of lethality (LD_{50}) was 100 PFU (Table 1). Different results were obtained in mice inoculated with each of the three T3A mutants. Indeed, no OFS was observed regardless of the dose of JH3 and JH4 used. In two of five experiments performed, all JH2-infected mice exhibited a few oily fur patches when inoculated with the highest dose of virus tested (10^6 PFU). We suspected that the appearance of these oily fur patches was due to the presence of revertants in the viral preparation, as previously described (Hooper and Fields, 1996). Of the three mutants examined, JH3 alone was consistently lethal and JH2 produced an occasional death. The dose of JH2 or JH4 required to produce 50% of lethality was at least 1000 times higher than the dose of T3A (Table 1).

These results showed that mutants JH2, JH3, and JH4 have lost their capacity to induce the OFS. The simplest interpretation of this result was that the different single point mutations identified in the M2 gene segment of these viruses were responsible for the loss of OFS. Nevertheless, it was possible that a mutation(s) affecting other gene segments may be involved. To address these questions, we analyzed the phenotype caused by reassortant viruses issued from a backcross between a representative T3A mutant, JH4, and reovirus EB144 (Hooper and Fields, 1996). EB144 is a reassortant virus containing seven T1L genes and three type 3 Dearing (T3D) genes, including the M2 gene and two nonstructural genes, S3 and M3 (Brown *et al.,* 1983). A backcross experiment performed with JH4 and this virus yielded reassortants containing different combinations of genes derived from both parents, including five viruses with a T1L S1 gene segment. These reassortants were not tested in mice since a T3A S1 gene is required for the OFS (Wilson *et al.,* 1994). Parental viruses JH4 and EB144 and reassortant EB144XJH4 or T3A (used as a positive control) were perorally inoculated into neonatal mice; OFS and lethality were measured. Parental viruses did not induce the OFS and were not lethal when inoculated at doses as high as 10^6 PFU (Table 2). In contrast, the LD_{50}s calculated for reassortants F204A.1, F175A, and F44A.2 were similar to those calculated for T3A (Table 2). No OFS was observed in mice infected with viruses containing a S1 and a M2 gene segment from JH4, namely, F204A.1 and F175A (Table 2). Furthermore, no intermediate phenotype was observed, even in mice inoculated with a high dose virus, 9×10^5 and 2×10^6 PFU, for F204A.1 and F175A, respectively. However, a clear and severe OFS was observed in mice infected with F44A.2, a reassortant virus containing a S1 gene segment from JH4 and a M2 gene segment from EB144 (Table 2). This phenotype was observed in mice inoculated with the lowest dose of F44A.2 tested, 3.3 PFU. F204A.1 and F175A possess a T1L L2, L3, S4 and a T1L L1, L2, L3, S4 from EB144, respectively. Thus, the combination of these T1L genes with a JH4 S1 gene did not restore the capacity of these viruses to cause the OFS, suggesting that these genes in JH4 do not bear a mutation that inhibited the OFS. In F44A.2, gene segments L2, L3, M1, S1, S2, and S4 are from JH4, indicating that they did not bear a mutation(s) that inhibited the S1 gene-associated OFS. In this virus, as is F204A.1 and F175A, S3 and M3 could be from either parental viruses. Consequently, no conclusion can be drawn concerning their association with the loss of OFS. Together, these results are consistent with previous data

TABLE 2

Lethality and Oily Fur Syndrome in Neonatal Mice Inoculated Perorally With JH4xEB144 Reassortant Viruses

	Genotypic mobility[c]											
	Outer capsid				Core				Nonvirion			
	S1	S4	M2	L2	S2	M1	L1	L3	S3	M3	Oily fur syndrome[a]	LD_{50}[b]
Parental viruses												
EB144	1L	1L	3D	1L	1L	1L	1L	1L	3D	3D	−	>6
JH4											−	>6
T3A											+	2.00
EB144XJH4 reassorants												
F204A.1	J	1L	J	1L	J	J	J	1L	−	−	−	3.77
F175A	J	1L	J	1L	J	J	1L	1L	−	−	−	2.77
F44A.2	J	J	3D	J	J	J	1L	J	−	−	+	2.00
Sum of exceptions[d]		3	0	3	1	1	1	3				

[a] Phenotype observed in mice ($n = 8$ to 10 per group). Mice were observed daily for oily fur development. Experiments were terminated at day 21 postinfection. (−) absence of oily fur; (+) severe oily fur covering the entire mouse and affecting the entire group of mice, including the mother.

[b] Number of PFU ($\log_{10}$) resulting in 50% mortality following po inoculation of 1- to 2-day-old Swiss Webster mice calculated by the method of Reed and Muench. The results represent data collected from one (for EB144) to three independent experiments.

[c] J, The genome segment runs with a JH4 mobility; 1L, the genome segment runs with a T1L mobility; 3D, the genome segment runs with a T3D mobility. In EB144XJH4, the parental origin of non virion protein encoding gene segments, S3 and M3, was not distinguishable by electropherotyping analysis.

[d] The sum of exceptions indicates the number of times a JH4 gene is associated with the OFS plus the number of times this gene derived from EB144 was associated with the loss of OFS.

showing a strong link between the biliary atresia-associated OFS and the T3A S1 gene segment (Wilson *et al.,* 1994). These results also demonstrate that a mutation in the central portion of the M2 gene segment can inhibit the OFS.

To further study the pathogenesis of the three T3A mutants, we inoculated 1- to 2-day-old mice with T3A ($n = 5$), JH2 ($n = 5$), JH3 ($n = 3$), or JH4 ($n = 5$) with a dose of 10^4 PFU or with distilled water (DW) ($n = 2$), as a control. Mice were sacrificed on day 10 postinfection when T3A-inoculated mice exhibited a severe OFS and livers were dissected for histopathological analysis. Mice infected with T3A showed evidence of severe jaundice; the liver and peritoneal cavity were typically yellow. In mice infected with JH2, JH3, or JH4, the appearance of the liver and peritoneal cavity was similar to those of uninfected mice. To evaluate the extent of tissue injury, two histologic sections of each liver, including one section at the level of biliary ducts, were made several millimeters apart and stained with hematoxylin and eosin. Blinded histopathological analysis was performed by Dr. M. Huerre (Département d'Histotechnologie et Pathologie, Institut Pasteur, France). Figure 1A shows an example of a liver tissue section from the control mice. In the liver of all T3A-infected mice examined, large foci of necrosis were observed and numerous infiltrated cells, monocytes and lymphocytes, were present in hepatic sinusoids surrounding portal arteries, venules, and biliary ducts (Figs. 1B and 1B′). Compared to the livers of T3A-infected mice, livers dissected from JH2-, JH3-, or JH4-infected mice exhibited a normal architecture with no or few pathological changes; some perivenular mononuclear cell infiltrates were present (Figs. 1C–1E, respectively).

DISCUSSION

In this study, we show that T3A viruses (JH2, JH3, JH4) containing three different single point mutations within the central portion of the M2 gene are strongly attenuated in their capacity to induce the OFS, a phenotype genetically linked to the T3A S1 gene segment. Genetic analysis using EB144XJH4 reassortants demonstrates that in JH4, the S1 gene does not bear a mutation(s) that altered the capacity of the σ1 protein to induce the OFS. Analysis of individual genes from each reassortant also demonstrated that neither L2 nor S4, which encode for proteins of the outer capsid playing major roles in the pathogenesis of animal infection (Fields and Byers, 1983; Sherry and Blum, 1994; Haller *et al.,* 1995) contains a mutation(s) that inhibited the OFS. A similar conclusion can be drawn for gene segments encoding for proteins of the viral core, particularly L1 and M1, that were previously linked to the capacity of reovirus T1L and 8B to damage specific tissues, such as the liver or the heart, inducing hepatitis in severe combined immunodeficiency mice (Haller *et al.,* 1995) or myocarditis in neonatal mice (Sherry and Blum, 1994), respectively. In contrast, the present results show that a mutation affecting the M2 gene segment could inhibit the σ1-associated OFS phe-

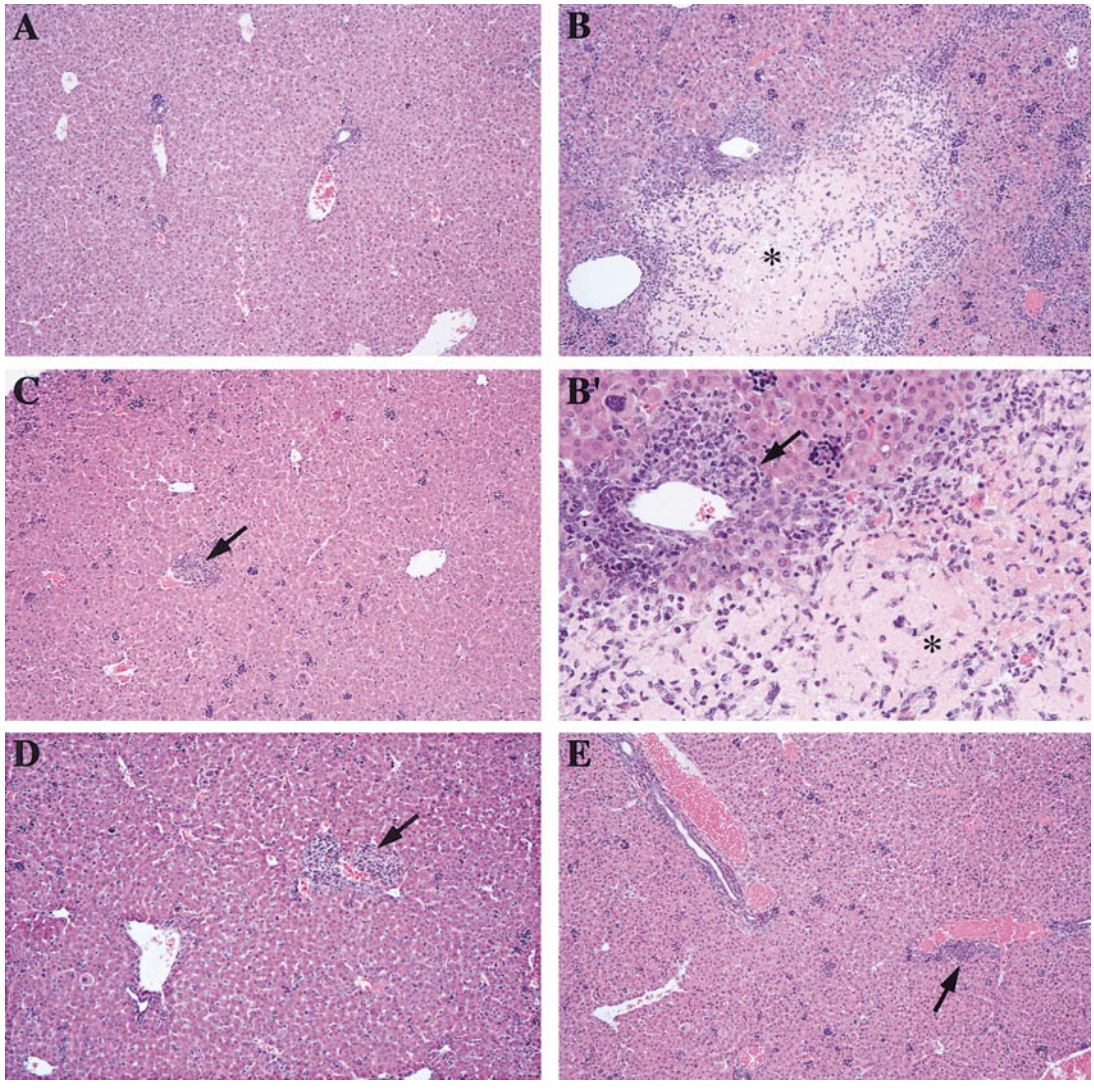

FIG. 1. Liver pathology following peroral infection. One- to two-day-old Swiss Webster mice were perorally inoculated with distilled water (A), T3A (B and B′), JH2 (C), JH3 (D), or JH4 (E) at a dose of 10^4 PFU. Mice were sacrificed at day 10 postinfection when T3A-inoculated mice exhibited a severe OFS and livers were dissected for histopathological analysis. (A) Liver section of a control mouse. (B and B′) Section of the liver of a mouse infected with T3A, showing extensive necrosis (*) and numerous mononuclear cell infiltrates (arrow) (original magnifications, ×100 and ×250, respectively, for B and B′). (C) Section of the liver of a mouse infected with JH2, showing few cell infiltrates (arrow) (original magnification, ×100). (D) Section of the liver of a mouse infected with JH3 showing no pathological change and few cell infiltrates (arrow). (E) Section of the liver of a mouse infected with JH4 showing no pathological change and few cell infiltrates (arrow) (original magnification, ×100).

notype since (i) viruses bearing a mutated M2 segment derived from JH4 did not induce the OFS and (ii) the replacement of this mutated M2 gene by a T3D M2 gene in a virus containing a JH4 S1 segment (F44.A.2) fully restores this phenotype. Because a monoreassortant containing a M2 gene segment from T3D in a JH4 background was not available, we cannot formally exclude the possibility that a mutation(s) affecting genes other than M2 may have a minor contribution and inhibit the T3A S1 gene-associated OFS or restore it when associated with a gene(s) of different parental origin. It might be the case for the two nonstructural proteins encoding gene segments, S3 and M3, although in animal models of infection, these two genes do not come out as major determinants of virulence (Fields and Byers, 1983; Haller *et al.,* 1995; Sherry and Blum, 1996). Moreover, no intermediate phenotype was observed in EB144XJH4 reassortant-infected mice, further supporting the idea that the loss of OFS is not due to a mutation(s) in several gene segments. Together, the results obtained with three different M2 mutants and with JH4xEB144 reassortants constitute strong evidence that a mutation affecting the central portion of the M2 gene segment in JH4, and also in JH2 and JH3, inhibits the S1 gene-associated OFS.

Histopathological analysis of the liver of mice inoculated with these T3A mutants shows that these mutant viruses cause essentially no necrotic damage in hepatic tissue, compared to wild-type T3A. The mechanisms

whereby T3A induces severe injury in this tissue remain largely unknown. Wilson and collaborators proposed that the OFS, a consequence of bile duct obstruction during T3A infection, could be related to the destruction of specialized epithelial cells associated with bile ducts (Wilson *et al.,* 1994). Thus, the lack of oily fur syndrome observed in mice infected with JH2, JH3, and JH4 could indicate that these viruses are not able to damage this epithelium. Previous findings showing that these mutants are altered in their capacity to permeabilize mouse L cells (Hooper and Fields, 1996), together with recent studies proposing a model in which both the type 3 S1 and M2 gene segment products induce apoptosis (Tyler *et al.,* 1996; Barton *et al.,* 2001), support this hypothesis. Alternatively, the conformation of the μ1/μ1C protein could determine the efficiency of the immune response to control reovirus infection in the liver. In agreement with this hypothesis, a previous study demonstrated that the M2 gene segment is involved in regulating the immune response in mice inoculated by the oral route (Rubin and Fields, 1981). Moreover, the OFS is observed in neonatal mice depleted in CD4 and CD8 T cells and infected with reovirus type 3 Clone 9 (Virgin and Tyler, 1991), a reovirus strain which does not induce oily fur (Wilson *et al.,* 1994). This suggests that T cells can protect liver cells against some reovirus infections, but not others (e.g., T3A). Thus, it is conceivable that the immune reaction initiated in response to infection with viruses containing a mutation in the M2 gene segment may be more efficient in protecting liver cells than the immune response induced by T3A.

In conclusion, the present results indicate that the M2 gene segment is involved in the capacity of reovirus T3A to cause hepatobiliary disease in mice and suggest that a mutation affecting the capsid protein, μ1/μ1c, could inhibit the capacity of reoviruses possessing the T3A cell attachment protein, σ1, to damage hepatic cells. This work brings new insights into the properties of the M2 gene segment product and may also contribute to the understanding of liver pathogenesis related to viral infection.

MATERIALS AND METHODS

Virus

Reovirus T3A, T3A mutants with a single point mutation within the central portion of the M2 gene (JH2 (residue 466 Tyr to Cys), JH3 (residue 459 Lys to Glu), and JH4 (residue 497 Pro to Ser) (Hooper and Fields, 1996)), EB144XJH4 reassortants (F44A.2, F175A, and F204A.1 (Hooper and Fields, 1996)) were grown from laboratory stocks. EB144 is issued from a backcross between T3D and T1L and contains a T1L S1 gene segment and a T3D M2 gene segment (Brown *et al.,* 1987).

Animal and inoculations

Pups born of pregnant Swiss Webster mice (Taconic Farms, Inc.) maintained in accordance with the standard guidelines were used in this study. Several litters of 1- to 2-day-old pups were randomized and groups of equal number were made. Mice were inoculated po with various doses of purified virions or second-passage virus stocks, freshly diluted in DW, as previously described (Wilson *et al.,* 1994; Derrien and Fields, 1999). Mice were observed daily for OFS development and lethality. Experiments were terminated at day 21 postinfection. For liver histology, viruses (or DW) were inoculated at a dose of 10^4 PFU po.

Tissue staining for liver histology

Tissue samples harvested at day 10 postinoculation were immersed in 4% neutral buffered Formalin (Fisher Scientific, Pittsburgh, PA), pH 7.4, for at least 24 h. Tissue sectioning and hematoxylin-and-eosin staining were performed by E. Meluleni in the animal core facilities at Harvard School of Public Health (Boston, MA).

Statistical analyses

The LD_{50} were calculated by the method of Reed and Muench (1938).

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