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Multiple Viral Core Proteins Are Determinants of Reovirus-Induced Acute Myocarditis

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Previously, we showed that the M1 gene (encoding a viral core protein, μ2, whose function is unknown) was associated with the efficiently myocarditic phenotype of a reovirus variant, 8B. Here, we have extended our genetic analysis of 8B and conducted genetic analyses of two other reovirus strains (T1L [serotype 1 strain Lang] and Abney). Our results demonstrate that multiple viral core proteins are determinants of reovirus-induced myocarditis. In contrast to our previous association of μ2 with induction of myocarditis, this provides strong evidence that a core function achieved through the interaction of multiple core proteins is responsible for induction of the disease.

Much evidence over the years suggests that a wide range of viruses are a significant cause of human myocarditis (24, 27, 38, 48). We are studying reovirus-induced murine myocarditis to identify the viral genes that determine the acute disease and to examine the mechanism by which the virus damages cardiac tissue. We have shown that efficiently myocarditic and poorly myocarditic reovirus strains replicate to similar titers in the heart (42, 44); thus, differences in myocarditic potential do not simply reflect growth in the heart. In primary cell cultures, the induction of cytopathic effect in cardiac myocytes, but not cardiac fibroblasts, is correlated with induction of myocarditis (3). Finally, cell-mediated immunity is important in protecting against the acute disease (43), which is consistent with the proposed mechanism for coxsackievirus-induced acute myocarditis (15), and in contrast to the suggested damaging role for immune components in coxsackievirus-induced chronic myocarditis (for reviews, see references 17 and 35). Thus, the acute disease most likely reflects a direct cytopathogenic effect of the virus on cardiac myocytes.

Our work has focused on an efficiently myocarditic reovirus isolate, 8B (42, 44). The efficiently myocarditic isolate 8B is a reassortant virus (i.e., contains a mixture of gene segments from two parent reovirus strains [for a review, see reference 46]), which was derived from a mouse coinfected with the poorly myocarditic strain serotype 1 strain Lang (T1L) and the nonmyocarditic serotype 3 strain Dearing (T3D) (44, 49). Previously, we had derived a panel of 31 reassortant viruses from genetic crosses between the efficiently myocarditic strain 8B and nonmyocarditic strains (42). We had then injected these reassortant viruses (2×10^5 to 2×10^6 PFU) into the left hindlimbs of NIH SW neonatal mice and examined their hearts for the presence of macroscopic external lesions (gross myocarditis) when the mice died or at 14 days postinjection (42). A Wilcoxon rank sum analysis of the results indicated that the M1 gene (encoding the viral core protein μ2, of unknown function [34, 47, 50]) was associated with induction of myocarditis at a highly significant level ($P = 0.002$), while the L2 gene (encoding the viral guanylyl transferase, λ2 [6, 26]) was implicated at low significance ($P = 0.05$) (42).

Because the original results formed a continuum of myocarditic potentials, we extended our studies by injecting most of the 31 reassortant viruses into neonatal mice at a higher dose (4×10^6 to 5×10^7 PFU) and then by examining their hearts as described above for gross myocarditis (data not shown). The results, in contrast to our previous results, were easily divided into two categories: nonmyocarditic and myocarditic (0 to 6% and 38 to 100% of the mice displaying gross myocarditis, respectively). However, interpretation of the results was the same. That is, Mann-Whitney analysis (identical to Wilcoxon rank sum analysis) indicated association of the same two genes with induction of gross myocarditis (the M1 gene at a highly significant level [$P < 0.001$] and the L2 gene to a lesser degree [$P = 0.039$]). We conducted one additional statistical analysis to identify viral genes associated with 8B-induced myocarditis. Given the strong association of the 8B-M1 gene with induction of myocarditis, we selected only reassortant viruses that contained the 8B-M1 gene and performed a Mann-Whitney analysis on this subset. This procedure eliminated the M1 gene requirement and therefore provided a sensitive assay for non-M1 genes associated with myocarditis. As expected, the 8B-L2 gene was implicated ($P = 0.05$); however, the 8B-L1 gene was also implicated ($P = 0.019$). Importantly, the M1, L1, and L2 genes from a given parent virus did not cosegregate in either the larger or the smaller group of reassortant viruses (by chi-square analysis). Thus, each of the three genes is associated with induction of the disease (i.e., none have been falsely implicated simply by their cosegregation with another gene).

Our genetic analyses of 8B-induced myocarditis were performed at a facility in which the parent virus T1L was only mildly myocarditic (gross myocarditis in approximately 10% of the mice) and the parent virus T3D was completely nonmyocarditic (44). We moved to new mouse facilities and found that both 8B and T1L had increased potentials to induce myocarditis (Fig. 1), while T3D continued to be nonmyocarditic even at high doses (Fig. 2). This difference did not reflect mouse source (National Cancer Institute), known pathogens (both colonies were continuously screened for common mouse pathogens), or environment (food, bedding, and light cycle), nor did it reflect virus stock, since the same virus stocks were used for injections at both facilities. Indeed, virus stocks were exchanged with researchers at the first facility, and their virus stocks demonstrated increased myocarditic potential at our new facility while our stocks displayed their former lower myocarditic potential at the first facility (45a). In addition,

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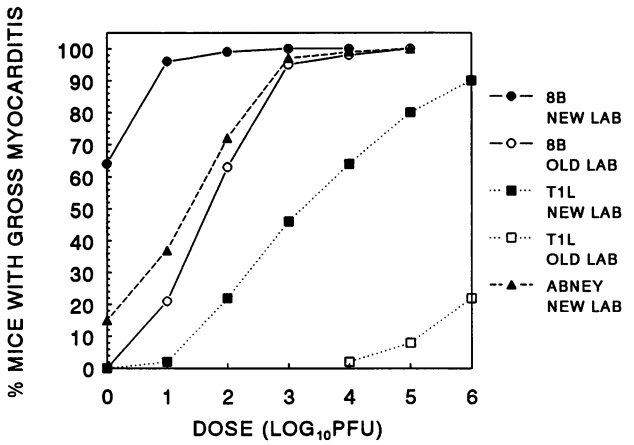

FIG. 1. Dose-response curves for frequencies of 8B- and T1L-induced gross myocarditis in two different mouse facilities (old and new lab) and Abney-induced myocarditis in the new facility (new lab). Neonatal mice were injected with the dose of virus indicated in the facility indicated. All 8B injections used a single virus stock, regardless of the facility, as did all T1L injections. Hearts (16 to 108 for each virus and dose tested) were examined when the animals died or at 14 days postinjection, and a heart was given a score positive if any external lesions were detected (gross myocarditis). Reed and Muench calculations were performed (33), and the results were plotted.

	GENE SEGMENT DERIVATION										% MICE WITH GROSS MYOCARDITIS	
	OUTER CAPSID			CORE					NON-STRUC			
VIRUS	S1	S4	M2	S2	M1	L1	L2	L3	S3	M3	%	N
T1L	1	1	1	1	1	1	1	1	1	1	60	(32/53)
T3D	3	3	3	3	3	3	3	3	3	3	0	(0/49)
EB145	1	3	3	3	3	3	3	3	3	3	0	(0/16)
G16	1	1	1	1	3	1	1	1	1	1	0	(0/18)
EB13	3	1	3	3	3	3	3	3	3	3	0	(0/16)
EB146	1	3	1	1	3	1	1	1	1	1	0	(0/16)
EB86	3	1	3	3	3	1	3	3	3	1	0	(0/16)
EB98	1	3	1	3	1	1	3	1	1	1	0	(0/21)
EB120	3	1	1	3	1	3	3	1	1	1	0	(0/17)
F17	1	1	1	3	1	1	3	3	1	1	0	(0/17)
G2	3	1	1	1	1	1	3	1	1	1	0	(0/20)
EB47	1	1	1	1	1	1	3	1	1	1	0	(0/19)
H15	3	1	3	3	1	1	3	3	3	3	4	(1/26)
EB1	1	1	3	1	1	1	3	1	3	1	6	(1/17)
EB138	3	1	3	3	1	3	1	1	1	1	6	(1/17)
H17	1	1	3	3	1	3	3	3	3	3	7	(2/27)
H14	1	1	1	1	1	1	1	3	3	1	9	(2/22)
EB85	1	1	1	3	1	1	1	1	1	3	11	(2/19)
H27	1	1	3	1	1	1	3	3	1	1	12	(2/17)
H41	1	1	1	3	1	3	3	3	3	3	12	(2/17)
EB136	3	3	3	3	1	3	3	3	3	1	13	(2/16)
EB68	1	3	3	1	1	1	3	1	3	1	20	(3/15)
EB143	3	1	1	1	1	3	1	1	1	1	20	(3/15)
EB93	1	1	1	1	1	1	1	1	3	1	24	(4/17)
EB39	3	3	3	3	1	1	3	3	3	3	24	(4/17)
H24	1	3	1	1	1	1	1	1	1	1	39	(11/28)
KC150	3	3	3	3	1	3	1	3	1	1	50	(8/16)
EB144	1	1	3	1	1	1	1	1	3	3	68	(13/19)
KC19	3	1	3	1	1	1	1	1	3	1	100	(16/16)

FIG. 2. Genetic analysis of T1L-induced myocarditis; frequency of gross myocarditis. Neonatal mice were injected with 10^6 PFU of virus, and their hearts were examined and scored as described in the legend to Fig. 1. Gene segment derivations are indicated in the following two ways: a black or white box indicates derivation from the myocarditic or nonmyocarditic parent, respectively, while 1 or 3 indicates the T1L or T3D origin of the gene segment. Mann-Whitney analysis indicates that only the M1 gene ($P = 0.006$) and the L2 gene ($P = 0.021$) are associated with induction of myocarditis (see text).

virus isolated from T1L-infected mice with hearts exhibiting gross myocarditis in the new facility proved to be T1L by genome analysis (data not shown). Nonetheless, this increased myocarditic potential did afford us the opportunity to use a panel of T1L-T3D reassortant viruses to identify T1L genes associated with the induction of myocarditis. We injected the 19 viruses (described in references 4, 7, and 9) into neonatal mice at 10^6 PFU per mouse and examined their hearts for gross myocarditis at day 14 postinjection or earlier if the mice were in visible distress (Fig. 2). Mann-Whitney analysis implicated two genes: the M1 gene ($P = 0.006$) and the L2 gene ($P = 0.021$). Again, chi-square analysis confirmed that these genes did not cosegregate in this panel of reassortant viruses (thus, neither was falsely implicated simply by its cosegregation with the other). In an attempt to detect other genes associated with induction of the disease, we selected only reassortant viruses that contained the T1L-M1 gene or the T1L-M1 and -L2 genes for Mann-Whitney analysis; however, no additional genes were implicated. The 8B-M1, -L1, and -L2 genes were derived from T1L; however, 8B is much more efficiently myocarditic than T1L (44). We are currently sequencing these genes to identify possible mutations.

In order to determine whether the association of the M1, L1, and L2 genes with myocarditis could be generalized to other reovirus-induced myocarditides, we initiated a similar study using an unrelated myocarditic reovirus serotype 3 strain, Abney. First, we generated a dose-response curve for Abney-induced gross myocarditis (Fig. 1). Abney induced myocarditis efficiently and was therefore suitable for use in a genetic analysis of the disease phenotype.

We derived a panel of 35 reassortant viruses from a genetic cross between the efficiently myocarditic strain Abney and a nonmyocarditic strain G16 (which was consistently nonmyocarditic in the new mouse facility [Fig. 2]). We injected these reassortant viruses into neonatal mice at two different doses (2×10^3 and 2×10^6 PFU per mouse, based on the dose-response curve of Abney in the new facility [Fig. 1]) and examined their hearts as described above for gross myocarditis. The results formed a continuum, with from 0 to 100% of the mice displaying gross myocarditis (displayed in Fig. 3 in order of increasing frequency, averaged between the two doses). Mann-Whitney analysis of results from medium-dose injections and low-dose injections identified three reovirus genes significantly associated with induction of gross myocarditis: the M1 gene ($P = 0.008$ and 0.004), the S1 gene ($P = 0.004$ and 0.006), and the L1 gene ($P = 0.016$ and 0.003). Again, chi-square analysis confirmed that these genes did not cosegregate in this panel of reassortant viruses (thus, none of them was falsely implicated simply by cosegregation with another gene).

The M1 gene was significantly associated with induction of gross myocarditis for each of the three myocarditic viruses, while the L1, L2, and S1 genes were associated with induction of the disease in a strain-dependent manner (Table 1). Interestingly, the same four genes have been linked to virulence in

VIRUS	GENE SEGMENT DERIVATION: OUTER CAPSID S1	S4	M2	CORE S2	M1	L1	L2	L3	NON-STRUC S3	M3	% MICE WITH GROSS MYOCARDITIS: MED DOSE %	MED DOSE N	LOW DOSE %	LOW DOSE N
ABNEY	A	A	A	A	A	A	A	A	A	A	100	(28/28)	96	(25/26)
G16	1	1	1	1	3	1	1	1	1	1	0	(0/18)	0	(0/8)
AG117	1	1	1	1	3	1	1	1	A	1	0	(0/17)	0	(0/21)
AG102	1	1	1	1	3	1	1	A	A	1	0	(0/10)	0	(0/8)
AG74	1	A	1	1	3	1	1	A	A	1	0	(0/9)	0	(0/10)
AG112	1	A	1	1	3	1	1	1	1	1	0	(0/10)	0	(0/9)
AG95	1	1	A	A	3	1	1	1	1	1	0	(0/19)	0	(0/15)
AG143	1	1	1	1	3	1	A	1	1	A	0	(0/17)	0	(0/19)
AG129	1	1	A	A	3	1	A	A	1	A	0	(0/11)	0	(0/9)
AG56	A	1	A	A	3	1	1	1	A	1	0	(0/18)	0	(0/7)
AG97	1	A	1	1	3	A	1	A	A	A	0	(0/20)	0	(0/22)
AG133	A	1	A	1	A	A	1	A	A	A	0	(0/17)	0	(0/18)
AG156	A	1	A	A	A	A	A	1	1	1	0	(0/19)	5	(1/20)
AG154	1	1	A	1	3	1	1	A	A	1	6	(1/18)	0	(0/18)
AG111	1	A	1	1	A	1	1	A	A	1	10	(3/31)	0	(0/9)
AG49	1	1	A	A	3	A	1	A	A	A	19	(4/21)	0	(0/19)
AG138	A	A	A	A	3	1	1	1	A	1	26	(5/19)	0	(0/9)
AG53	1	1	1	A	3	A	1	1	1	1	24	(4/17)	7	(1/14)
AG88	1	1	1	1	A	A	1	1	1	1	27	(9/33)	15	(5/33)
AG69	A	A	A	1	3	A	1	1	A	1	33	(6/18)	14	(2/14)
AG118	1	A	1	1	A	A	A	A	A	1	25	(4/16)	33	(7/21)
AG106	1	1	A	1	A	1	1	1	1	1	47	(9/19)	40	(8/20)
AG107	1	1	1	1	A	A	1	1	1	1	48	(14/29)	44	(16/36)
AG113	A	A	1	1	A	A	A	A	A	A	85	(17/20)	11	(2/19)
AG145	A	A	1	1	A	1	A	A	A	1	88	(14/16)	11	(2/18)
AG71	1	A	A	1	A	A	1	1	A	A	53	(9/17)	58	(18/31)
AG116	1	1	A	1	A	A	1	A	1	1	94	(16/17)	37	(7/19)
AG80	A	1	A	A	A	A	A	A	A	A	95	(18/19)	71	(5/7)
AG124	A	A	A	1	A	A	1	A	A	A	100	(19/19)	78	(7/9)
AG150	A	A	A	1	A	A	A	A	A	1	100	(9/9)	82	(14/17)
AG68	A	1	1	A	A	A	1	1	A	A	85	(17/20)	100	(10/10)
AG139	1	A	A	1	A	1	1	1	1	A	100	(15/15)	89	(8/9)
AG72	A	A	A	1	A	A	A	A	A	1	95	(19/20)	100	(11/11)
AG63	A	1	1	A	3	A	1	1	A	A	100	(17/17)	100	(9/9)
AG78	A	A	A	1	3	A	1	A	A	A	100	(29/29)	100	(8/8)
AG54	A	1	1	A	3	A	1	1	1	1	100	(18/18)	100	(9/9)
AG90	A	1	A	A	A	1	A	A	1	A	100	(17/17)	100	(15/15)

FIG. 3. Genetic analysis of Abney-induced myocarditis; frequency of gross myocarditis. Neonatal mice were injected with either 2×10^6 PFU (med dose) or 2×10^3 PFU (low dose), and their hearts were examined and scored as described in the legend to Fig. 1. Gene segment derivations are as described in the legend to Fig. 2 (A indicates derivation from Abney). Mann-Whitney analyses of results from medium- and low-dose injections indicate, respectively, that only the M1 gene ($P = 0.008$ and 0.004), the S1 gene ($P = 0.004$ and 0.006), and the L1 gene ($P = 0.016$ and 0.003) are associated with induction of myocarditis (see text).

severed combined immunodeficient mice (15a). The M1 gene encodes a viral core protein, μ2, whose function is unknown (34, 47, 50). The L1 gene encodes the viral polymerase λ3 (10, 45), the L2 gene encodes the viral guanylyl transferase λ2 (6, 26), and the S1 gene encodes the viral attachment protein σ1 (22), as well as a second protein whose function is unknown (12, 19, 36). Electron microscopy (14, 32) and recent cryoelectron microscopy (11, 31, 31a) suggest that the M1, L1, L2, and S1 protein products are found in intimate association at the icosahedral vertices of the virus (Fig. 4). Electron micrographs of transcriptionally active viral cores extruding nucleic acid from channels at the core vertices (1) suggest that the replication complex may synthesize early transcripts inside the core at the vertices and cap them as they are extruded through the

TABLE 1. Summary of gene segments associated with induction of acute myocarditis

Myocarditic virus	Gene segment: Outer capsid S1	S4	M2	Core S2	M1	L1	L2	L3	Nonstruc S3	M3
8B					+	+	+			
T1L					+		+			
Abney	+				+	+				

guanylyl transferase channel (the σ1 obstruction has been removed by this point). μ2 may play a role in this process (or in later RNA synthesis) by nature of an arginine-rich motif reminiscent of RNA-binding proteins (5, 21). Indeed, recent evidence suggests that μ2 is associated with viral RNA synthesis in certain cell lines (29).

None of the four genes was required for induction of the disease, suggesting that it is the interaction between these proteins that determines the disease. Indeed, many individual viruses which lacked the 8B-L1 or -L2 gene (42 and data not shown), the T1L-L2 gene (Fig. 2), or the Abney-S1, -M1, or -L1 gene (Fig. 3) were myocarditic. These data suggest that any of a number of gene combinations among the four genes can result in myocarditis. A much larger panel of reassortant viruses would be required to assess the impact of each possible protein-protein interaction on the phenotype (and then one might find evidence of cosegregation of genes). Interestingly, two viruses (AG133 and AG156 [Fig. 3]) contained all three Abney genes (Abney-S1, -M1, and -L1) associated with the induction of myocarditis; however, they did not induce the disease. These data suggest either that other reovirus genes are involved in the induction of myocarditis or that AG133 and AG156 have suffered attenuating mutations.

How could these proteins function in the induction of myocarditis? We propose two hypotheses.

First, myocarditic strains may have altered interactions among the four proteins affecting their RNA synthetic functions. We have shown that viral yield from cultured cardiac myocytes is determined by the M1 and L1 genes, while viral yield from mouse L cells is determined only by the L1 gene (30), which is consistent with our proposed role for a cell-specific effect of μ2 on RNA synthesis. Altered timing or rates

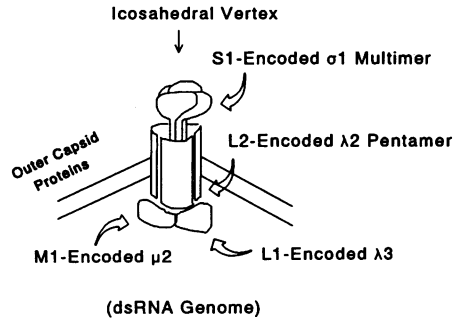

FIG. 4. Location of the M1-, L1-, L2-, and S1-encoded proteins in the reovirion. μ2 and λ3 are entirely inside the core, λ2 (as a pentamer [31]) extends from the core through the outer capsid, and a multimer of σ1 (2, 13, 23) is partially surrounded by the λ2 pentamer and partially exposed (14). The locations of these proteins are based on cryoelectron microscopic data (see references 11, 31, and 31a). dsRNA, double-stranded RNA.

of transcription of reovirus genes which affect host cell macromolecular synthesis (25, 28, 39–41) could profoundly alter host cell metabolism and induce a cytopathic effect (8). This cytopathic effect would also result in efficient spread through the tissue. The observed specificity for cardiac myocytes compared with cardiac fibroblasts (3) may reflect different sensitivities of these cells to effects of reovirus on macromolecular synthesis.

Second, the interactions among these four proteins may be altered in myocarditic strains, affecting reovirus core stability and induction of interferon. Evidence suggests that the reovirus genome does not fully uncoat during replication (for a review, see reference 37), perhaps to avoid exposure of its double-stranded RNA genome, which is a potent inducer of interferon (for reviews, see references 20 and 37). Some reovirus strains do induce interferon, perhaps because of a leaking of the genome (16). Myocarditic strains might possess very stable cores and thus induce very little interferon. A low level of interferon induction would result in efficient spread through the infected tissue. The observed differences in the cytopathic effect in cardiac myocytes and cardiac fibroblasts could reflect differences in their induction of and/or sensitivity to (18) interferon.

These data provide new insight into reovirus-induced acute myocarditis, in that multiple viral proteins intimately associated in the viral core are all implicated in induction of the disease. In contrast to our previous association of μ2 with induction of myocarditis, this provides strong evidence that a core function achieved through the interaction of multiple core proteins is responsible for induction of the disease.

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