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Genetic Mapping of Reovirus Virulence and Organ Tropism in Severe Combined Immunodeficient Mice: Organ-Specific Virulence Genes

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We used reovirus reassortant genetics and severe combined immunodeficient (SCID) mice to define viral genes important for organ tropism and virulence in the absence of antigen-specific immunity. Adult SCID mice infected with reovirus serotype 1 strain Lang (T1L) died after 20 ± 6 days, while infection with serotype 3 strain Dearing (T3D) was lethal after 77 ± 22 days. One hundred forty-five adult SCID mice were infected with T1L, T3D, and 25 different T1L × T3D reassortant reoviruses, and gene segments associated with the increased virulence of T1L were identified. Gene segments S1, L2, M1, and L1 accounted for >90% of the genetically determined increase in T1L virulence. Gene segment M1 was independently important for virulence, with S1, L2, and L1 alone or in combination also playing a role. T1L grew to higher titers in multiple organs and caused more severe hepatitis than T3D. Seventy adult SCID mice, T1L, T3D, and 15 T1L × T3D reassortant viruses were used to map genetic determinants of viral titers in the brain, intestines, and liver, as well as the severity of hepatitis. Different sets of gene segments were important for determining viral titers in different organs. Gene segments L1 (encoding a core protein) and L2 (encoding the core spike of the virion) were important in all of the organs analyzed. The M1 gene segment (encoding a core protein), but not the S1 gene segment, was a critical determinant of reovirus titer in the liver and severity of hepatitis. The S1 gene segment (encoding the viral cell attachment protein and a nonstructural protein), but not the M1 gene segment, was a critical determinant of titers in intestines and brains. These studies demonstrate that viral growth in different organs is dependent on different subsets of the genes important for virulence. The virion-associated protein products of the four gene segments (L1, L2, M1, and S1) important for virulence and organ tropism in SCID mice likely form a structural unit, the reovirus vertex. Organs (the brain and intestines versus the liver) differ in properties that determine which virulence genes, and thus which parts of this structural unit, are important.

Reoviruses are nonenveloped viruses containing 10 double-stranded RNA gene segments which cause a variety of diseases in neonatal mice (reviewed in reference 33). When different reoviruses infect the same cell, the 10 gene segments reassort, allowing selection of progeny viruses with different combinations of parental gene segments. Nine of 10 gene segments encode a single protein (segment S1 encodes two proteins; reviewed in reference 24). By studying phenotypes of parental and reassortant progeny viruses, gene segments, and therefore proteins, which define pathogenetic phenotypes can be identified.

Immunologic studies have shown that antibody and CD4 and CD8 T cells play a role in controlling reovirus infection in neonatal mice (27, 34, 36, 37, 39). This raises the possibility that antigen-specific immunity plays a role in determining which gene segments are important in pathogenesis in neonatal mice. More recently, severe combined immunodeficient (SCID) mice lacking functional T and B cells have been used to study reovirus pathogenesis (13, 27). While central nervous system (CNS) disease is prominent in neonatal mice, adult SCID mice develop lethal systemic reovirus infection associated with severe hepatitis. In contrast, normal adult mice are not susceptible to lethal infection (13, 31). We decided to study reovirus pathogenesis in adult SCID mice since the SCID model allows analysis of gene segments involved in organ tropism and virulence in adult mice in the absence of antigen-specific immunity.

In this report, we show that prototypic reoviruses T1L and T3D differed significantly in the rates at which they killed SCID mice (virulence). Four gene segments, including three encoding components of the viral core, were associated with differences in reovirus virulence in SCID mice. Further analysis showed that all four of these gene segments are involved in determining virus titers in organs and/or severity of hepatitis in SCID mice (measures of organ tropism) but that different combinations of gene segments are important in different organs.

MATERIALS AND METHODS

Viruses and tissue culture. Mouse L929 cells were maintained in a spinner culture with minimal essential medium supplemented with 5% fetal calf serum (Hyclone, Ogden, Utah, or Biofluids, Rockville, Md.), 2 mM L-glutamine (Biofluids), and 100 U of penicillin per ml plus 100 μg of streptomycin per ml (Biofluids or Irvine Scientific, Santa Ana, Calif.) (MEM^+). T1L and T3D and T1L × T3D reassortant viruses were from laboratory stocks originally the gift of B. Fields and M. Nibert and have been used extensively in other studies (3, 5, 8, 9, 16, 26, 35). Viruses were plaque purified twice and passaged twice to increase

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the titers before inoculation into mice. The methods used for virus growth, storage, and plaque assay on L929 cells were previously described (34, 36, 37, 39).

Mice, mouse inoculations, and organ harvesting. Adult SCID mice on the CB17 background were maintained by serial brother-sister mating at the Washington University School of Medicine. Mice were housed at biosafety level 2 in accordance with all federal and university policies. Sentinel mice were used to rule out infection with adventitious mouse pathogens. Infection of SCID mice with reovirus elicited no reovirus-specific antibody, even in SCID mice having detectable serum immunoglobulin G (unpublished observation). To define the natural history of reovirus infection in SCID mice, adult male and female SCID mice 1 to 7 months of age were inoculated intraperitoneally (i.p.) with 1 ml of MEM^+ containing 4×10^5 to 5.7×10^7 PFU of T3D or reassortant reoviruses per mouse (see Table 2). T1L at doses of 4×10^4 to 1.1×10^7 PFU per mouse was used to assess the dose dependence of virulence in SCID mice. Animals were observed daily, and the day of death was recorded. For assessment of titers in organs; adult male and female SCID mice 1 to 4 months of age were inoculated i.p. with 2.2×10^6 to 2.4×10^7 PFU of reovirus. Mice were sacrificed 12 days after inoculation, and organs were harvested into 1.0 ml of gel saline (37). Organs included a piece of liver (1 by 1 cm), 1.5 to 2 cm of the proximal small bowel from the gastric antrum through the duodenum, and the posterior two-thirds of the brain. Organ samples were frozen and thawed three times prior to sonication and plaque assay. Data are presented as the mean $\log_{10}$ titer per milliliter $\pm$ the standard error of the mean.

Histopathology. Liver from mice used to determine viral titers at day 12 (see above) was fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Slides were coded to hide the identity of the sample and scored by H.W.V. The number of inflammatory lesions, defined as lesions containing five or more inflammatory cells, per 200× field was scored. At least 30 randomly selected 200× fields were evaluated to determine the mean number of lesions per field.

Statistical analysis. Both nonparametric and parametric methods were used to ensure that conclusions were independent of the statistical method used. The nonparametric Wilcoxon test was performed by ranking the mean day of death, mean virus titer in an organ, or mean number of hepatic lesions per field for groups of mice receiving different viruses. We then tested for effects of parental origin of each gene segment on the pathogenetic endpoint with $P \leq 0.05$ as significant. Parametric multiple regression analysis used individual mouse survival time, virus titer in an organ, or number of hepatic lesions per microscopic field as the dependent variable predicted by parental origin of the 10 reovirus gene segments. The proportion of variance (R^2) for a pathogenetic endpoint predicted by the parental origin of each gene segment was determined. We defined $R^2 > 0.15$ as significant, since contributions of variables such as viral dose and mouse age were $R^2 \leq 0.01$ to 0.12 across all of our experiments. Second, a pairwise analysis of gene segments was performed to determine if information from one gene segment added significantly to information from a second segment. We used the conservative criteria that a gene segment must have a high R^2 and consistently add information to other gene segments in pairwise analyses to firmly establish an association between a phenotype and the parental origin of a gene segment. A third method assessed the independent contribution of a gene segment to the variance predicted by a set of other gene segments. We established the total R^2 for a set of gene segments (either all 10 gene segments [data not shown] or the most significant subset of gene segments [Table 6]) and then subtracted information from individual gene segments one at a time and assessed whether a decrease in the prediction of variance occurred. Results obtained with all 10 gene segments and selected subsets of gene segments were similar. All analyses were conducted with the SAS (1).

RESULTS

Virulence of T1L, T3D, and T1L × T3D reassortant reoviruses in SCID mice. Twenty-two adult SCID mice in six experiments died 14 to 35 (average, 20 $\pm$ 6) days after i.p. infection with T1L (Table 1 and Fig. 1). In contrast, 12 mice in three experiments infected i.p. with T3D died 24 to 105 (average, 77 $\pm$ 22) days after infection. The kinetics of lethal infection were comparable in different experiments with the same dose of T1L or reassortant reoviruses H24 and EB18 (data not shown; see Table 2 for identification of reassortant viruses). Varying the infecting dose of virus over a 25-fold (T3D), 38-fold (EB39), or 142-fold (T1L) range had little effect on the kinetics of lethal infection (Table 1). T1L killed SCID mice more rapidly than T3D, even when the dose of T1L was lower than that for T3D (compare experiments 3 and 6 in Table 1). Defining virulence as the rapidity with which reoviruses kill adult SCID mice, T1L was significantly more virulent than T3D. Virulence was dependent primarily on the genetic makeup of viruses as opposed to the infecting dose.

TABLE 1. Reovirus infection in adult SCID mice[a]

Expt	Virus	Dose (PFU/mouse)	No. of mice	Avg no. of days alive
1	T1L	5.7×10^6	5	21
2	T1L	4×10^5	4	17
3	T1L	4×10^4	3	25
4	EB39	1.5×10^7	4	38
5	EB39	4×10^5	4	43
6	T3D	9.8×10^6	5	85[b]
7	T3D	4×10^5	4	89

[a] Adult SCID mice were injected i.p. with the virus doses indicated, and mortality was monitored. The average number of days a mouse survived was calculated by adding the total number of survival days for a group and dividing by the number of experimental animals. Results of three of six representative experiments are shown for T1L. Results of two of three representative experiments are shown for T3D.

[b] Four of five mice were sacrificed on day 87 because of illness, and thus this is an underestimate of the average number of days alive for this group.

A panel of T1L × T3D reassortant reoviruses (containing different combinations of T1L and T3D gene segments; Table 2) was used to define gene segments associated with virulence in SCID mice. We performed six experiments (2 to 22 adult SCID mice per group, a total of 145 SCID mice) in which 25 reovirus reassortants plus T1L and T3D were injected i.p. and mouse mortality was monitored (Fig. 1). Reassortants fell into two groups. One group of reassortants killed SCID mice rapidly, with most mice dead before day 60 (Fig. 1, viruses 1 to 19 [virus numbers in Fig. 1 refer to those in Table 2]). A second group of reassortants killed SCID mice with kinetics similar to those of T3D (Fig. 1, viruses 20 to 27). No reassortant virus was significantly more virulent than T1L, and few viruses killed SCID mice as rapidly as T1L. Thus, the presence of T3D gene segments in the T1L background decreased virulence. For example, reassortant G16 (virus 11 in Fig. 1) has all of the T1L gene segments except M1 from T3D, and reassortant G2 (virus 17 in Fig. 1) has all of the T1L gene segments except S1 and L2 from T3D. Both G16 and G2 are less virulent that T1L.

Nonparametric analysis of associations of specific gene segments with virulence. Using survival data for groups of SCID mice infected with T1L, T3D, and T1L × T3D reassortants (Fig. 1), we calculated the average number of days a mouse survived after infection with each reovirus. These values were ranked (Table 2), and Wilcoxon analysis was performed. Four gene segments, M1, L1, L2, and S1, were associated with virulence (P values, 0.02 to 0.001; Table 3). Similar analyses ranking viruses by first day of death or day when 50% of the mice died showed statistical significance for the same four gene segments (data not shown). One possible reason for the association of four gene segments with virulence was nonrandom distribution of T1L and T3D gene segments in the reassortants used in this analysis (Table 2). We did a pairwise chi-square analysis of M1, L1, L2, and S1 by parental origin and found significant ($P < 0.05$) associations between pairs of all four T1L gene segments except the pair M1 and S1 (data not shown). These nonrandom associations reflect the limited availability of certain combinations of gene segments within the available pool of reassortants (see Discussion). Wilcoxon analysis allowed us to conclude that some combination of these four gene segments is important for determining virulence in SCID mice.

Regression analysis of associations of specific gene segments with virulence. Multiple regression analysis showed that 70% of the variance ($R^2 = 0.7$) in reovirus virulence in SCID mice was attributable to the 10 reovirus gene segments. Thus,

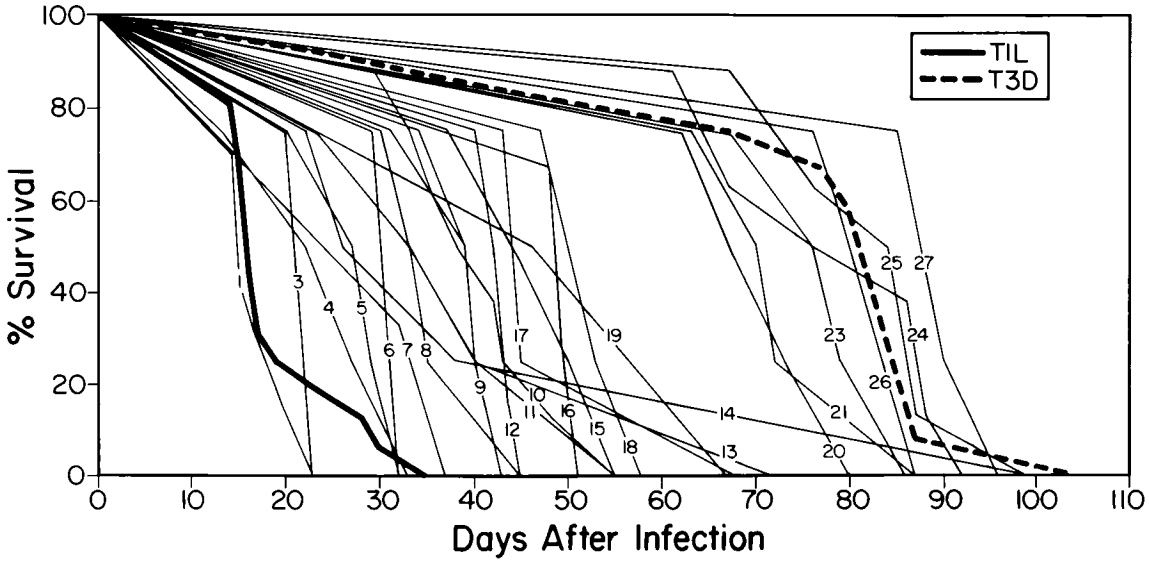

FIG. 1. Natural history of parental and reassortant reovirus infection after i.p. infection of SCID mice. Numbers on lines refer to numbers of viruses in Table 2. Data from all experiments using either T1L (six experiments) or T3D (three experiments), including those in Table 1, were used to generate the parental curves shown here. The thick solid line is pooled data for T1L and the thick dashed line is pooled data for T3D. All groups had at least four mice, except viruses 7 and 16 ($n = 3$) and virus 19 ($n = 2$).

TABLE 2. Reovirus reassortants used to map gene segments involved in virulence and organ tropism in SCID mice

No., virus	Origin[a] of gene segment encoding:										Avg no. of days alive	Rank
	OC[b]/NS[c] protein	OC protein		Core spike	Core				NS protein			
	S1	S4	M2	L2	S2	M1	L1	L3	S3	M3		
1, H24	1	3	1	1	1	1	1	1	1	1	17	1
2, T1L	1	1	1	1	1	1	1	1	1	1	20	2
3, EB1	1	1	3	3	1	1	1	1	3	1	22	3
4, EB144	1	1	3	1	1	1	1	1	3	3	24	4
5, EB85	1	1	1	1	3	1	1	1	1	3	27	5
6, H14	1	1	1	1	1	1	1	3	3	1	31	6
7, EB47	1	1	1	3	1	1	1	1	1	1	34	7.5
8, H17	1	1	3	3	3	1	3	3	3	3	34	7.5
9, EB109	1	3	3	3	3	3	3	1	3	3	37	9
10, EB39	3	3	3	3	3	1	1	3	3	3	40	10
11, G16	1	1	1	1	1	3	1	1	1	1	41	11
12, EB136	3	3	3	3	3	1	3	3	3	1	43	12
13, F17	1	1	1	3	3	1	1	3	1	1	46	13
14, EB68	1	3	3	3	1	1	1	1	3	1	47	14.5
15, EB31	1	1	1	1	3	3	1	1	3	1	47	14.5
16, EB121	1	3	1	3	3	3	3	1	3	3	49	16
17, G2	3	1	1	3	1	1	1	1	1	1	50	17
18, EB146	1	3	1	1	1	3	1	1	1	1	52	18
19, EB13	3	1	3	3	3	3	3	3	3	3	57	19
20, EB108	1	3	1	3	1	3	1	1	3	1	71	20
21, H30	3	3	3	3	3	3	3	3	1	3	73	21
22, T3D	3	3	3	3	3	3	3	3	3	3	77	22.5
23, EB145	1	3	3	3	3	3	3	3	3	1	77	22.5
24, EB18	1	1	3	3	1	3	3	1	3	3	78	24
25, EB120	3	1	1	3	3	1	3	3	1	3	83	25.5
26, EB129	3	3	3	3	1	3	3	1	1	1	83	25.5
27, EB86	3	1	3	3	3	3	1	3	3	1	90	27

[a] Origins of gene segments: 1, TIL; 3, T3D.
[b] OC, outer capsid.
[c] NS, nonstructural.

the primary determinant of virulence in our experiments was viral genetic makeup. We performed the following tests to ensure that variables other than the parental origin of gene segments did not confound our analyses of gene segment associations with virulence. Mouse sex and age and viral dose were evaluated and found to contribute minimally to virulence (R^2 for age plus dose plus sex equals 0.11). To rule out an untoward contribution of a mutation affecting virulence in an individual reassortant, we showed that deletion of data from each reassortant virus one at a time did not significantly change our conclusions (data not shown).

Table 4 shows the results of regression analysis of reovirus gene segment associations with virulence in SCID mice. In agreement with Wilcoxon analysis, S1, L2, M1, and L1 were the best predictors of virulence (compare Tables 3 and 4). We

TABLE 3. Wilcoxon analysis of reovirus gene segments involved in virulence, virus titer in organs, and severity of hepatitis

Protein[a] encoded	Gene segment	P value for association with[b]:				
		Virulence	Titer in brain	Titer in intestine	Titer in liver	Liver lesions
OC/NS	S1	0.01	0.001	0.001		
OC	S4					
OC	M2					
CS	L2	0.01	0.005	0.001	0.05	
Core	S2					
Core	M1	0.001			0.005	0.01
Core	L1	0.02	0.02	0.005	0.02	0.05
Core	L3			0.02		
NS	S3					
NS	M3					

[a] OC, outer capsid; NS, nonstructural; CS, core spike.
[b] An empty cell means $P > 0.05$.

TABLE 4. Regression analysis of reovirus gene segments involved in virulence, titers in organs, and severity of hepatitis

Protein(s)[a] encoded	Gene segment(s)	R^2 for prediction of variance in:				
		Virulence	Titer in brain	Titer in intestines	Titer in liver	Liver lesions
All 10	All 10	0.70	0.78	0.94	0.67	0.66
OC/NS	S1	0.28	0.59	0.68	0.16	0.17
OC	S4	0.05	0.11	0.16	0.13	0.04
OC	M2	0.14	0.10	0.19	0.06	0.02
CS	L2	0.38	0.40	0.63	0.30	0.25
Core	S2	0.15	0.06	0.14	0.04	0.10
Core	M1	0.37	0.15	0.16	0.39	0.33
Core	L1	0.35	0.39	0.51	0.31	0.18
Core	L3	0.18	0.18	0.38	0.10	0.07
NS	S3	0.07	0.01	0.00	0.01	0.03
NS	M3	0.11	0.03	0.08	0.01	0.01

[a] OC, outer capsid; NS, nonstructural; CS, core spike.

performed pairwise analyses to determine if the parental origin of a second reovirus gene segment added information to that obtained from each gene segment alone. For example, we defined the R^2 for S1 and then assessed whether adding information from other gene segments improved the prediction of variance (Table 5, right-side columns). We found that information from L2, M1, and L1 each improved the prediction of variance by S1. Knowing the parental origin of M1 improved the prediction of variance by eight of nine other gene segments. By using this pairwise analysis for all gene segments, we found that the S1, L2, M1, and L1 gene segments frequently added information to the prediction of virulence obtained from other gene segments. This result confirms that S1, L2, M1, and L1 are significant for virulence and demonstrates that this significance is not due to nonrandom associations of gene segments within reovirus reassortants (Table 2).

We next evaluated the S1, L2, M1, and L1 gene segments as a group. We found that 64% of the total variance ($R^2 = 0.64$) in virulence was attributable to these four gene segments (Table 6). Therefore, since 70% ($R^2 = 0.70$) of the variance in virulence is attributable to all 10 gene segments (Table 4), 91% (0.64/0.70 × 100%) of the variance attributable to viral genetic makeup was accounted for by S1, L2, M1, and L1. We then evaluated whether these gene segments were independently important for predicting variance in virulence. We calculated the change in variance when information from individual gene segments was subtracted from the R^2 for all 10 gene segments (data not shown) or from the R^2 for S1 plus L2 plus M1 plus L1 (Table 6). This conservative approach demonstrated that M1 independently contributed to virulence. The S1 and L2 segments alone had some independent effect on virulence, but the effect was less profound than that of M1. The S1, L2, and L1 gene segments, either individually or in combination with each other or with M1, contribute 78% of the variance attributable to the combination of S1 plus L2 plus M1 plus L1 ($R^2 = 0.64$ for all four − $\Delta R^2 = 0.14$ for M1/$R^2 = 0.64$ for all four × 100%).

TABLE 5. Proportion of variance (R^2) in virulence predicted by individual gene segments and added contribution of a second segment

Protein[a] encoded	Gene segment	R^2	Added contribution of second gene segment[b]									
			S1	S4	M2	L2	S2	M1	L1	L3	S3	M3
OC/NS	S1	0.28				0.16		0.29	0.17			
OC	S4	0.05	0.24			0.33		0.32	0.30	0.17		
OC	M2	0.14	0.17			0.24		0.26	0.22			
CS	L2	0.38						0.20				
Core	S2	0.15	0.15			0.24		0.28	0.21			
Core	M1	0.37	0.20			0.21				0.16		
Core	L1	0.35										
Core	L3	0.18				0.22		0.35	0.21			
NS	S3	0.07	0.26			0.31		0.30	0.29			
NS	M3	0.11	0.19			0.28		0.30	0.24			

[a] OC, outer capsid; NS, nonstructural; CS, core spike.
[b] An empty cell means an added contribution of <0.15.

Comparison of virus titers in organs and severity of hepatitis in SCID mice infected with T1L, T3D, and T1L × T3D reassortants. To understand better how different reoviral gene segments contribute to virulence, we evaluated titers of T1L, T3D, and a panel of 15 T1L × T3D reassortants in organs of adult SCID mice. Mice were infected i.p. with different viruses, and organs were harvested on day 12 for titer determination (Fig. 2A to C). T1L grew to higher titers than T3D in the liver, intestines, heart (data not shown), and brain. T1L also grew to higher titers than T3D in the liver, intestines, and brain 5, 15, and 20 days after infection (data not shown). Importantly, the differences in replication we found between T1L and T3D in SCID mice are specific to the in vivo situation. In L929 cells, T1L and T3D grew equally well both 14 and 24 h after infection (data not shown). By using reassortant viruses, we observed a broad range of titers in different organs, with a consistent trend toward more virulent viruses having higher titers in multiple organs (compare Fig. 1 and 2). However, patterns of reassortant growth were different in different organs. For example, both virus 10 (EB39 in Table 2) and virus 17 (G2 in Table 2) grew in the liver as well as viruses with similar virulence. In contrast, growth in the intestines and brain was less than that of viruses with similar virulence. This suggested that the determinants of viral titer might differ depending on the organ evaluated. Since previous work demonstrated the presence of severe hepatitis in T1L-infected SCID mice (13), liver sections were scored for numbers of inflammatory lesions (Fig. 2D). T1L caused more severe hepatitis (3.07 ± 0.19 lesions per field) than did T3D (0.56 ± 0.13 lesions per field).

Associations of specific gene segments with virus titers in organs and severity of hepatitis. We used Wilcoxon and regression analyses to evaluate genetic determinants of virus titers in organs and severity of hepatitis (Fig. 2 and Tables 3 and 4). Regression analysis showed that variables including virus dose and mouse sex and age contributed less than 20% of the observed variance in viral titers in different organs. In contrast, the 10 reovirus gene segments determined most of the variance in viral titers in organs (67 to 94%; Table 4). To ensure that gene segment associations with virus titers in organs were not biased by the fact that we selected fewer reassortants for evaluation of viral titer and pathology (Table 2 and Fig. 2) than we used to evaluate virulence (Table 2 and Fig. 1), we performed Wilcoxon analysis of gene segment associations with virulence by using the subset of viruses used for titer analysis. All four gene segments associated with virulence in the larger set of reassortants were significantly

TABLE 6. Effect of removing variance due to individual gene segments on prediction of variance in pathogenetic endpoints by different combinations of gene segments

Segment subtracted	Change in R^2 for combinations of gene segments after subtracting information from individual gene segments S1, L2, M1, and L1[a]				
	Virulence, S1 + L2 + M1 + L1 (R^2 = 0.64)	Titer in brain, S1 + L2 + L1 (R^2 = 0.72)	Titer in intestines, S1 + L2 + L1 (R^2 = 0.9)	Titer in liver, L2 + M1 + L1 (R^2 = 0.57)	Liver lesions, L2 + M1 (R^2 = 0.47)
S1	0.05	0.24	0.19		
L2	0.04	0.01	0.05	0.08	0.14
M1	0.14			0.20	0.22
L1	0.00	0.07	0.06	0.01	

[a] An empty cell means not applicable.

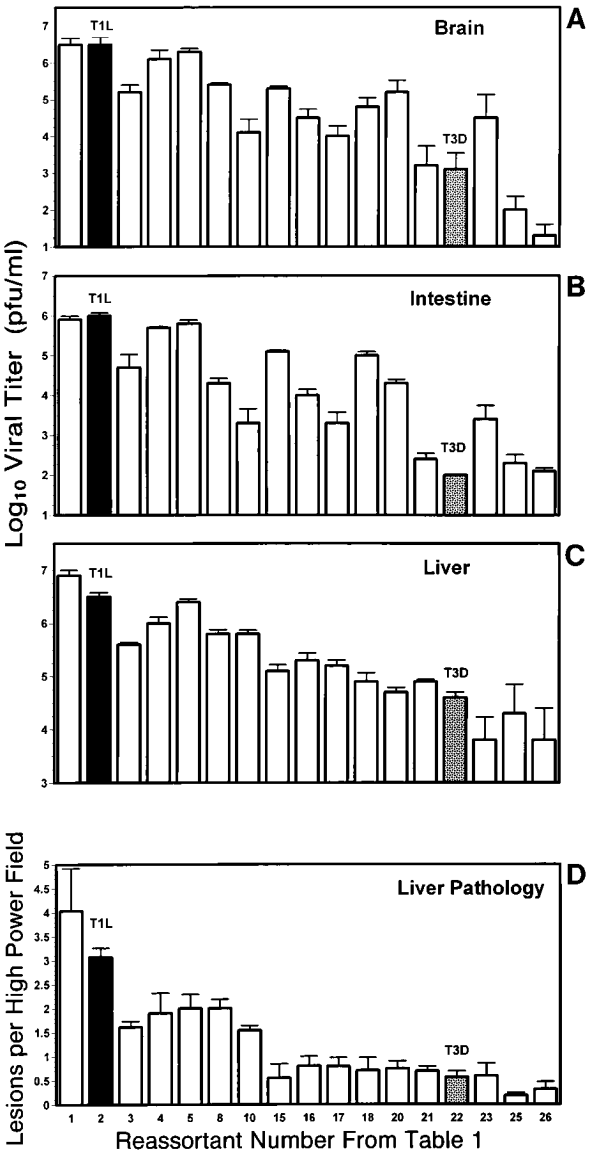

FIG. 2. Reovirus titers in multiple organs and severity of hepatitis in SCID mice. SCID mice were infected i.p. with different viruses, and the viral titers in the brain (A), intestines (B), and liver (C) were determined 12 days later. The livers used for titer determination were evaluated histopathologically in a blinded fashion to determine the number of inflammatory lesions per field (D). Numbers on the abscissa refer to numbers of viruses in Table 2. Viruses are numbered from more virulent (virus 1) to less virulent (virus 26).

associated with virulence when we used data from the smaller set of reassortants used for analysis of growth in different organs (data not shown).

Both Wilcoxon and regression analyses revealed that different gene segments were predictive of viral titers in different organs (Tables 3 and 4). Gene segments L1 and L2 were consistently associated with viral titers in all of the organs tested. However, gene segment M1 was predictive of the titer in the liver but much less important in the brain and intestines. Conversely, gene segment S1 was predictive of the titers in the brain and intestines but was much less important in the liver. When the severity of hepatitis was evaluated, we found that gene segment M1 had the strongest association with the lesion number (Tables 3 and 4) and that S1 played, at most, a minimal role. We further assessed the roles of different gene segments in determining virus titers in organs by performing a pairwise analysis of the capacity of individual gene segments to improve the prediction of variance by other gene segments (data not shown). For the brain and intestines, information from gene segments S1, L2, and L1 consistently added information to other gene segments while the M1 gene segment failed to provide additional information consistently. In contrast, L2 and M1 (with a lesser effect of L1) consistently improved the prediction of variance for both the titer in the liver and the severity of hepatitis, while the S1 gene segment failed to provide additional information consistently. The parental origin of gene segments other than S1, L2, M1, and L1 failed to improve the prediction of variance in virus titers in organs or severity of hepatitis consistently (data not shown).

We determined the proportion of variance in titers in organs predicted by limited subsets of viral genes (Table 6). For the intestines and brain, the combination of L1, L2, and S1 allowed prediction of 92 and 98% of the genetically determined variance in virus titers tissues, respectively. For example, for the virus titer in the brain, $R^2 = 0.72$ (Table 6) for L1 plus L2 plus S1, while $R^2 = 0.78$ (Table 4) for all 10 gene segments ([0.72/0.78] × 100% = 92%). Removal of the contribution of S1 had a significant effect on the prediction of variance (Table 6) in viral titers in both the brain and intestines, while removal of L2 and L1 had smaller effects. In contrast to the situation in the brain and intestines, the combination of L2 and M1 predicted 66% of the total variance in the viral titer in the liver and 71% of the variance in the severity of hepatitis. In these cases, removal of the contribution of the M1 gene segment had a significant impact on the prediction of variance while removal of L2 had a more modest effect.

DISCUSSION

We used reassortant genetics to analyze determinants of viral pathogenesis in SCID mice. Differences in reoviral virulence (rapidity of death after infection) and organ tropism

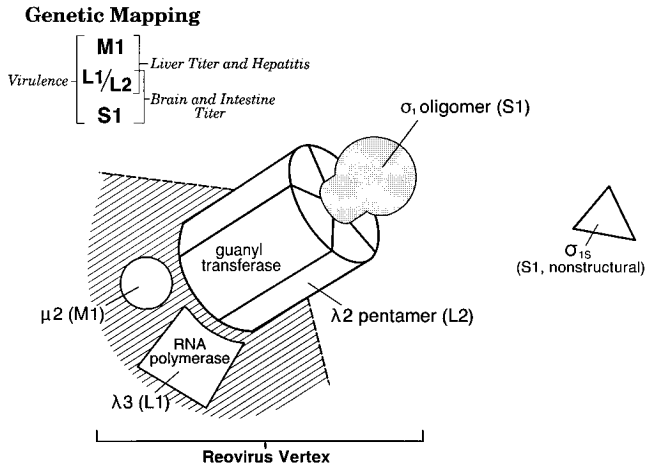

FIG. 3. Schematic representation of the likely arrangement of the reovirus vertex, with protein designations followed by the gene segments encoding the proteins in parentheses. In addition, the gene segment associations with individual pathogenetic endpoints are summarized.

(viral titers in organs and severity of hepatitis) between prototypic reoviruses T1L and T3D were due primarily to gene segments S1, L2, M1, and L1. In aggregate, these segments encode proteins found at the vertices of the reovirion (Fig. 3). Of these segments, M1 was the most important independent determinant of virulence. The relative importance of individual gene segments in determining virus titers in tissues depended on the organ (Fig. 3). The S1 gene segment was a key determinant of virus titers in the brain and intestines, with the M1 gene segment playing a minimal role. In contrast, the M1 gene segment was a critical determinant of both virus titers in the liver and severity of hepatitis, with the S1 gene segment playing a minimal role. These findings demonstrate (i) the value of SCID mice for genetic analysis of viral virulence, (ii) that virulence and organ tropism in SCID mice are controlled by gene segments whose virion-associated protein products likely form a structural unit (the viral vertex [Fig. 3; see below]), and (iii) that different organs, or sets of organs (the brain and intestines versus the liver), share properties that determine which viral genes are most important in determining organ tropism. Of special note is the fact that the same genes were important for both CNS tropism and intestinal tropism. These organs, which share limited organizational or functional similarity, are critically important for the pathogenesis of a number of important human viruses, including picornaviruses and retroviruses.

Gene segments involved in virulence and organ tropism in SCID mice likely encode proteins which form a structural unit. Reoviruses have an icosahedral outer protein capsid surrounding a protein core which contains and replicates the viral double-stranded RNA genome segments after uncoating in infected cells (4, 28). The vertex of the reovirion has been characterized in some detail (Fig. 3). The S1 gene segment encodes outer capsid protein σ1 and a nonstructural protein with an unknown function (11, 15, 23). σ1 is the cell attachment protein and is present as an oligomer at the 12 vertices of the reovirus capsid, where it associates with the core spike (10, 12, 17, 30, 42). The core spike is a pentamer of the λ2 protein encoded by the L2 gene segment (reviewed in reference 10). The λ2 pentamer extends from the surface of the virion into the core and is therefore available for interactions with both outer capsid protein σ1 and core proteins present at the bases of the viral vertex (reviewed in reference 10). In addition to forming the core spike, λ2 likely participates in RNA synthesis as a guanyl transferase (7, 18). The M1 and L1 gene segments both encode minor core proteins (μ2 and λ3, respectively) and are present in approximately 12 copies per virion (reviewed in reference 10), consistent with an association between μ2 and λ3 and each of the 12 reovirus vertices. In addition, cryoelectron microscopy studies (9a, 10), suggest that λ3 and, possibly, μ2 are intimately associated with the base of the viral vertex and therefore with λ2. In fact, λ3 physically associates with complexes of λ2 and core protein λ1 (29). μ2 has no currently recognized biochemical function (22, 43, 44). λ3 has RNA polymerase activity (29) but likely requires other core proteins to generate template specificity. Thus, the virion-associated protein products of the four gene segments which we defined as determinants of pathogenesis in SCID mice likely form a structural unit, the vertex of the reovirion. Known or likely functions of this unit include provision of structural integrity, RNA synthesis (involving at least products of L1 and L2), and cell tropism-attachment involving the σ1 product of the S1 gene segment.

Why are different gene segments important in different organs? Perhaps the most striking observation we made is that the importance of certain gene segments to determination of organ tropism depends on the organ. As the products of these gene segments likely form a structural unit, we believe that different parts of the viral vertex are more or less important, depending on the tissue evaluated. Some gene segments (L1 and L2) were important regardless of the organ analyzed. In contrast, M1 was important in the liver, while S1 was important in the intestines and brain. One model that may explain this finding is sequential involvement of different gene segment products in reovirus infection, with the rate-limiting step in viral growth or clearance differing from organ to organ. For example, if the S1 gene segment is important because it encodes the cell attachment protein, the nature of cells infected in the CNS or intestines could be the most important determinant of titers in the CNS or intestines (the rate-limiting step). The M1 gene segment product would, in this model, provide a function downstream from cell attachment and thus not be limiting in the intestines and CNS. Under this model, the S1 gene product would not be limiting in the liver (both the T1L and T3D S1 gene segment products serve equally well) but the M1 gene segment product would provide a rate-limiting function in viral replication or clearance. An alternate model would be that the host response controlling viral replication is distinct in different organs. Perhaps tissue-specific cytokines exist, or the importance of host factors, such as alpha/beta interferon, is different in different organs. In this model, the specific viral gene segment which is important for determining the viral titer in tissue would be involved in the step(s) in viral replication which is targeted by tissue-specific host factors.

Genetic determinants of reovirus pathogenesis in adult SCID versus neonatal outbred mice. L2, S1, and M1 have important pathogenetic roles in outbred neonatal mice, as well as adult SCID mice. The S1 gene segment determines routes of spread to and cellular tropism within the CNS (32, 35, 40, 41). Both S1 and L2 play a role in viral growth and survival in the intestines after oral inoculation (3), and L2 is a determinant of spread by the fecal-oral route (16). M1 determines the myocarditic potential of reovirus 8B (26). More recent data, based on the use of several different crosses, have shown that the same four gene segments identified in this report determine the myocarditic potential of various reoviruses in neonatal mice (25). The M2 gene segment is important for reduced neurovirulence within serotype 3 reoviruses (14) but had no

role in our studies. Our failure to find an important role for M2 may reflect the different reassortant crosses used for our studies and those defining M2 (6), differences in experimental systems, or a critical role for M2 obscured by the T1L and T3D M2 segments serving equally well in adult SCID mice. Thus, in general, the same gene segments are important in both neonatal outbred and adult SCID mice. This is somewhat surprising since reovirus-induced disease is different in adult SCID and outbred neonatal mice. For example, T1L kills adult SCID mice faster than does T3D (Fig. 1) while T3D is much more virulent than T1L in neonatal mice (the intracranial 50% lethal dose of T3D is 10 PFU, compared with greater than 10^7 PFU for T1L [34, 37]). In addition, while neonatal mice develop prominent encephalitis (T3D) or hydrocephalus (T1L), adult SCID mice develop prominent hepatitis (this report and reference 13) with limited neuropathology (unpublished observation).

Role of the S1 gene segment in virulence and virus titers in tissue. The S1 gene segment, encoding the cell attachment protein, is the primary determinant of cell tropism and route of spread to and within the CNS (35, 41) in neonatal mice. Thus, a likely explanation for the importance of S1 in determining viral titers in SCID mouse intestines and brains is that T1L σ1 allows infection of or spread between cells, which T3D σ1 does not. Alternatively, the mapping of the S1 gene as important for growth in the intestines and brain might be due to the nonstructural product of the S1 gene segment. It is interesting that T1L S1 confers the capacity to infect and damage ependymal cells of the CNS, while T3D S1 confers tropism for neurons (41). It is possible that the importance of T1L S1 in both the brains and intestines of SCID mice reflects preferential replication in neurons or neuron-associated cells in both the intestines and brain. It will therefore be interesting to identify the cell types infected by T1L, T3D, and T1L $\times$ T3D reassortants in both SCID mouse intestines and brains.

Role of the M1 gene segment in virulence and titers in tissues. The M1 gene segment was independently important for determining reovirus virulence, titer in the liver, and severity of hepatitis. This is consistent with the observation that the most impressive pathologic change, and likely cause of death, in adult SCID mice infected with reoviruses is hepatitis (13). The contribution of the M1 gene segment to virulence is therefore most likely due to its role in the liver. The M1 gene segment is also associated with the severity of myocarditis caused by myocarditic reovirus 8B in neonatal mice (25, 26). In myocarditis caused by reovirus 8B, the contribution of M1 is not explained by an increased titer of reovirus 8B compared with less myocarditic viruses in the heart. This has led to the proposal that reovirus 8B (and the M1 gene segment) causes myocarditis because it efficiently kills cardiac myocytes (2). In adult SCID mice, M1 was important for both titers in tissue and severity of hepatitis. Despite differences between neonatal mice and adult SCID mice, the M1 gene segment consistently contributes to the severity of tissue injury. The protein product of M1 gene segment μ2 has no defined function. It is possible that the μ2 protein itself is toxic to certain cells. However, since μ2 is likely a part of the reovirus vertex and one role of the vertex is viral RNA synthesis, we favor the hypothesis that the M1 gene product has a role in determining host response or cytopathicity via an undefined enzymatic activity in RNA transcription.

Contributions of the L1 and L2 gene segments to viral virulence and titers in tissue. The L1 and/or L2 gene segments were statistically associated with viral virulence and titer in every organ by both Wilcoxon and regression analyses. However, pairwise analysis of the capacity of information from L1 to improve the prediction of variance based on L2 or vice versa showed that the contributions of L1 and L2 were frequently confounded. In 9 of 10 cases (Table 5 and data not shown), neither addition of information from L1 to L2 nor addition of information from L2 to L1 improved the prediction of variance in viral titers in organs, virulence, or severity of hepatitis. Independent contributions of gene segments which are nonrandomly distributed in our reassortants were detected in other cases. For example, the L2 and M1 gene segments are nonrandomly distributed by parental origin in the reassortant panel used to map viral virulence and titers in organs, but L2 consistently contributes to prediction of variance by M1 and M1 contributes to prediction of variance by L2, except for titers in the brain and intestines, where M1 has no significant role (data not shown).

Our inability to detect independent effects of L1 and L2 consistently may be related to the fact that the products of L1 and L2 are structurally and functionally interrelated. Both have enzymatic activities associated with RNA synthesis. Thus, the T3D L1-L2 gene product complex and the T1L L1-L2 gene product complex may each function better than complexes containing mixtures of the T3D and T1L proteins. The idea of interdependence of function between two proteins which physically interact was first emphasized in the analysis of revertants of reovirus temperature-sensitive mutants. Revertants of temperature-sensitive mutants often map to gene segments encoding proteins which physically interact with the protein containing the original temperature sensitivity mutation (19–21). Additional support for coevolution of proteins physically or functionally associated with one another is provided by the demonstration that epitopes on reovirus capsid proteins σ3 and μ1 are preserved within a serotype, even though the serotype is determined by the σ1 protein (38). Further evidence for a close relationship between L1 and L2 comes from the observation that reassortants selected in L cells show nonrandom association of T1L L1 and L2 and T3D L1 and L2 (3, 5, 7a, 8, 9, 16, 26, 35). For example, in the set of reassortants from which we drew our experimental viruses, the T1L L2 gene segment is not found together with the T3D L1 gene segment except when there is either a mutation in the L1 gene product or altered mobility of a gene segment encoding another core protein (7a). Thus, interdependence of function of the L2 and L1 gene products could explain why we found it difficult to dissociate the influence of L1 and that of L2.

As the L2 and L1 gene products both have enzymatic activities associated with RNA transcription (see above), our data are consistent with a role for RNA synthesis in determining virulence and/or growth efficiency in SCID mice. Since T1L and T3D grow equally well in L cells in vitro, any differences between the RNA synthetic machinery of T1L and that of T3D are not important in tissue culture. It is possible that the efficiency of L2 and L1 protein products is not limiting in a cell line but is limiting in cells in vivo. For example, a transformed cell could provide substrates or protein factors missing in primary cells. In this scenario, the function of T1L L2-L1 products, and therefore aspects of RNA synthesis, are predicted to be limiting in vivo.

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