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Derivation and Characterization of an Efficiently Myocarditic Reovirus Variant

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A reovirus variant, 8B, was isolated from a neonatal mouse which had been inoculated with a mixture of two reovirus strains: type 1 Lang (T1L) and type 3 Dearing (T3D) (E. A. Wenske, S. J. Chanock, L. Krata, and B. N. Fields, J. Virol. 56:613–616, 1985). 8B is a reassortant containing eight gene segments derived from the T1L parent and two gene segments derived from the T3D parent. Upon infection of neonatal mice, 8B produced a generalized infection characteristic of many reoviruses, but it also efficiently induced numerous macroscopic external cardiac lesions, unlike either of its parents. Microscopic examination of hearts from infected mice revealed myocarditis with necrotic myocytes and both polymorphonuclear and mononuclear cellular infiltration. Electron microscopy revealed viral arrays in necrotic myocytes and dystrophic calcification accompanying late lesions. Determination of viral titers in hearts from T1L-, T3D-, or 8B-infected mice indicated that growth was not the primary determinant of myocardial necrosis. Results from inoculations of athymic mice demonstrated that T cells were not a requirement for the 8B-induced myocarditis. Finally, 8B was more cytopathic than either of the parent viruses in cultured mouse L cells. Together, the data suggest that 8B-induced myocardial necrosis is due to a direct effect of reovirus on myocytes. Reovirus thus provides a useful model for the study of viral myocarditis.

Reoviruses, members of the family *Reoviridae*, have a genome composed of 10 double-stranded RNA gene segments. They produce a generalized infection in neonatal mice, which can result in a variety of diseases (for a review, see reference 31). Specific virus strains are responsible for characteristic diseases; for example, type 3 Dearing (T3D) causes a lethal encephalitis, whereas type 1 Lang (T1L) causes an ependymitis in the brain. Comparisons between reovirus strains which cause different diseases have been useful in identifying viral determinants responsible for such disease patterns. We have recently isolated a particularly virulent reovirus variant which produces not only the disease patterns characteristic of its parents but also a striking myocarditis, unlike its parents. Certain reovirus strains have been reported to be efficiently myocarditic in mice (10, 33), but those studies did not include any comparisons between myocarditic and nonmyocarditic strains and thus provided no insight into differences which might determine the disease phenotype. The fortuitous isolation of an efficiently myocarditic reovirus variant in our laboratory has provided us with an opportunity to conduct such a comparison.

Myocarditis is generally characterized by myocardial necrosis in the presence of an inflammatory infiltrate composed of lymphocytes, monocytes, and/or polymorphonuclear cells (1, 17). Although myocarditis is often assigned an unknown etiology, viruses have been suspected for many years as the primary agent responsible for this important human disease (36). Reoviruses are ubiquitous in humans, although their connection to human disease is largely undefined. The observed myocarditis in mice suggests that reovirus may be a candidate for the human disease. Coxsackieviruses, on the other hand, have been directly implicated in human myocarditis (2). In an effort to understand the mechanism(s) underlying the generation of this disease, myocarditic and nonmyocarditic coxsackievirus variants have been studied in the mouse extensively over the past decade, but there has been little progress in understanding why some coxsackieviruses are efficiently myocarditic while others are silent (see Discussion). Using reoviruses instead, we present here (i) the derivation of our myocarditic variant, (ii) a comparison with its nonmyocarditic parents, (iii) a morphological characterization of the lesions, and (iv) preliminary investigations into the possible mechanism underlying the myocarditic phenotype of the variant.

MATERIALS AND METHODS

Virus stocks and cells. Mouse L929 (L) cells were propagated as Spinner cultures in the Joklik modification of Eagle minimal essential medium supplemented with 2 mM glutamine, 1 U of penicillin per ml, 1 μg of streptomycin (Irvine Scientific, Santa Ana, Calif.) per ml, and either 5% fetal calf serum (Hyclone Laboratores, Logan, Utah) or 2.5% fetal calf serum and 2.5% viable serum protein (agamma VSP; Biocell, Carson, Calif.) (completed minimal essential medium). Viruses were triply plaque purified and then passaged twice (to increase virus stock titer) on mouse L cells. The second-passage (P2) virus stocks were clarified by low-speed centrifugation before titration and use. Virus stocks were stored at 4°C. All gene segment derivations were confirmed by polyacrylamide gel electrophoresis of viral double-stranded RNA as described elsewhere (28). T1L and T3D were from standard laboratory stocks (20).

Mice, inoculations, and specimen removal. Pregnant NIH Swiss (NIH/Sw) mice and pregnant *nu*/+ and +/+ BALB/cAnNCR mice were purchased from the National Cancer Institute (Frederick, Md.), housed in individual filter-topped

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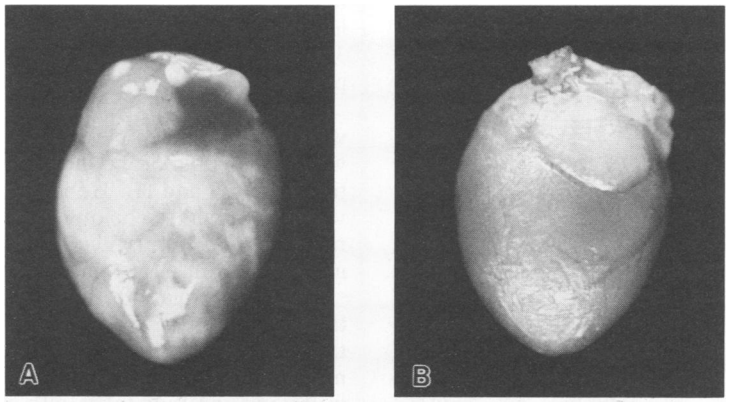


FIG. 1. Hearts from 8B-infected mice with numerous macroscopic external lesions. Neonatal NIH/Sw mice were injected intramuscularly in the left hindlimb with 10^6 PFU of 8B (A) or with gel saline (B). Hearts were removed 7 days postinjection and photographed immediately. T1L- and T3D-infected hearts looked like mock-infected hearts (data not shown).

cages, and checked daily for births. Neonates (2 days old) were inoculated in the left hindlimb with 20 μl of virus diluted in gel saline (137 mM NaCl, 0.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 19 mM H_3BO_3, 0.1 mM $Na_2B_4O_7$, 0.3% [wt/vol] gelatin [Fisher, Medford, Mass.]) by using a 1-ml tuberculin syringe and a 30-gauge needle. Offspring of *nu*/+ mice were confirmed as *nu*/+ or *nu*/*nu* by 3 to 5 days of age by the presence or absence of fur. On the indicated day postinjection, mice died or were sacrificed by cervical dislocation, and the hearts were removed and examined for the presence of macroscopic external cardiac lesions. The hearts were disposed of or treated as described below for plaque assays, histology, or electron microscopy.

Histology. Hearts for histopathologic examination were immediately immersed in 10% phosphate-buffered Formalin (Fisher). They were mounted as transverse sections, embedded in paraffin, sectioned to 6 μm, and stained with hematoxylin and eosin (16). For quantification of the fraction of myocardium involved in lesions, midcardiac sections were scored blindly as follows. Sections were viewed at ×125 magnification through a reticle imprinted with a grid of 1-mm squares. The number of intercepts falling over a necrotic region was expressed as a percentage of the total number of intercepts examined. Each cardiac section was viewed in its entirety, and, depending on the age of the mouse, 800 to 1,200 intercepts were scored for each section. One section from each of 6 to 11 mice was scored for each virus on each of the days indicated. The results were analyzed by Student's *t* test.

Electron microscopy. Hearts were minced into approximately 1-mm pieces by using a razor blade, fixed immediately in Karnovsky fixative (0.1 M cacodylate-buffered 2.5% glutaraldehyde, 2% paraformaldehyde, pH 7.2), postfixed in OsO_4, dehydrated, and embedded in Poly/Bed 812 medium (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JSM-100; JEOL, Nutley, N.J.).

Plaque assays. Hearts were minced into approximately 1-mm pieces by using a razor blade and placed in 2 ml of gel saline. Samples were frozen (−70°C) and thawed (37°C) three times and then sonicated for 15 to 30 s by using a microtip probe at 50% maximal setting (W225R Cell Disruptor; Heat Systems-Ultrasonics Inc., Plainview, N.Y.). The virus suspensions were serially diluted in 10-fold steps in gel saline and plated in duplicate on L-cell monolayers for plaque assays as described previously (32). Viral titers were expressed as PFU per heart. Determinations of viral titers in the blood from 8B-, T1L-, or T3D-infected mice on days 1 to 9 postinjection with 10^4 PFU indicated that a maximum of 10^2 PFU was present in the volume of blood contained in each heart (data not shown).

Measurement of cytopathic effect. Mouse L cells were seeded at a density of 10^6 cells per 16-mm tissue culture well (Costar 24-well tissue culture dishes) in 1 ml of completed minimal essential medium and allowed to attach for 2 to 4 h at 37°C. Overlying medium was aspirated off, and monolayers were inoculated (in triplicate) with 100 μl of gel saline containing enough virus to result in the indicated multiplicity of infection. After 1 h on ice, 1 ml of 37°C completed minimal essential medium was added, and the plates were incubated for 22 h at 37°C. Monolayers were dispersed by using a Pasteur pipette, cell suspensions were mixed 1:1 with trypan blue (0.4% solution; catalog no. T9520, Sigma Chemical Co., St. Louis, Mo.), and cell viability was determined by exclusion of trypan blue. The results were analyzed by Student's *t* test.

RESULTS

Generation of reassortant 8B in vivo. Neonatal NIH/Sw mice were inoculated perorally with a mixture of T1L and T3D reovirus, and reassortant viruses were recovered (34). One isolate recovered from the brain on postinoculation day 13, called 8B, was selected for further study. It was triply plaque purified and then passaged twice on mouse L cells to increase the stock titer. Of its 10 gene segments, 8 were derived from the T1L parent, and 2 (the S1 and M2 gene segments) were derived from the T3D parent (28).

8B displays a novel phenotype in vivo. When 8B was used to inoculate neonatal NIH/Sw mice perorally, intraperitoneally, or intramuscularly, it exhibited a phenotype distinct from that of either of its parents, T1L and T3D. Hearts removed from mice 7 days postinjection with 10^6 PFU of 8B displayed striking external macroscopic lesions, evident as white stripes and patches covering most of the surface of the heart (Fig. 1A). In contrast, hearts from mice injected with T1L or T3D had no visible lesions and were otherwise indistinguishable from hearts removed from uninfected mice (Fig. 1B and data not shown). All studies described in this report used intramuscular injections.

In order to confirm that this 8B phenotype was truly distinctive, we injected mice with a range of doses of either

TABLE 1. Frequency of macroscopic external cardiac lesions

Dose (log_{10} PFU) and virus	No. of mice with macroscopic external cardiac lesions[a]/no. examined on days postinjection:		
	5 to 8	9 to 14	15 to 21
3.0 to 3.8			
T3D	0/16	0/22	0/4
T1L	0/12	1/6	1/10
8B	11/25	36/36	1/2
4.0 to 4.8			
T3D	0/13	0/10	0/11
T1L	0/24	2/16	2/16
8B	23/28	23/23	0/1
5.0 to 6.0			
T3D	0/27	0/27	0/2
T1L	1/28	3/28	3/20
8B	51/55	8/8	0/1

[a] Mice died or were sacrificed during the periods indicated. A heart was scored as positive if it exhibited even one macroscopic external lesion.

8B, T1L, or T3D and then examined their hearts for the presence of macroscopic external lesions at various days postinjection (Table 1). These doses of 8B resulted in the death of most mice between 8 and 12 days postinjection (data not shown); therefore, data for later time points for 8B could not be obtained. 8B induced external macroscopic lesions in most mice even at low doses of virus (10^3 to 10^4 PFU), whereas external lesions were absent after T3D injection at any dose. T1L-injected mice did occasionally display external heart lesions; however, they appeared as only a few external stripes, in contrast to the extensive lesions induced by 8B (data not shown). Moreover, the frequencies of mice displaying external cardiac lesions were much greater for 8B infection that for T1L infection at comparable virus doses and times postinjection.

External macroscopic heart lesions are correlated with pancarditis upon microscopic examination. We removed hearts from mice 3, 5, or 7 days postinjection with 10^6 PFU of 8B, T1L, or T3D and selected midheart sections for microscopic examination (Fig. 2). Lesions in animals receiving 8B were the most extensive and pronounced (see below).

The earliest 8B-induced lesions examined (3 days postinjection) were characterized by myocyte vacuolization, hypereosinophilia, nuclear pyknosis, edema, and sparse polymorphonuclear leukocytic infiltration (Fig. 2A and B). Lesions at 5 days postinjection were larger and consisted of central myocyte necrosis and loss of sarcoplasm, with polymorphonuclear leukocytic inflammation and focal mononuclear inflammation (Fig. 2C and D). The periphery of the lesions showed changes similar to those of the earliest lesions described for day 3 postinjection. At 7 days postinjection, the necrotic lesions were again larger and consisted of a central zone of pronounced necrosis with extensive dystrophic myocyte calcification and a rim of less-pronounced necrosis with focal polymorphonuclear and mononuclear inflammation (Fig. 2E and F). The diameters of these lesions varied from less than approximately 200 to 300 μm at 3 days postinjection to 400 to 600 μm or more at 5 and 7 days postinjection.

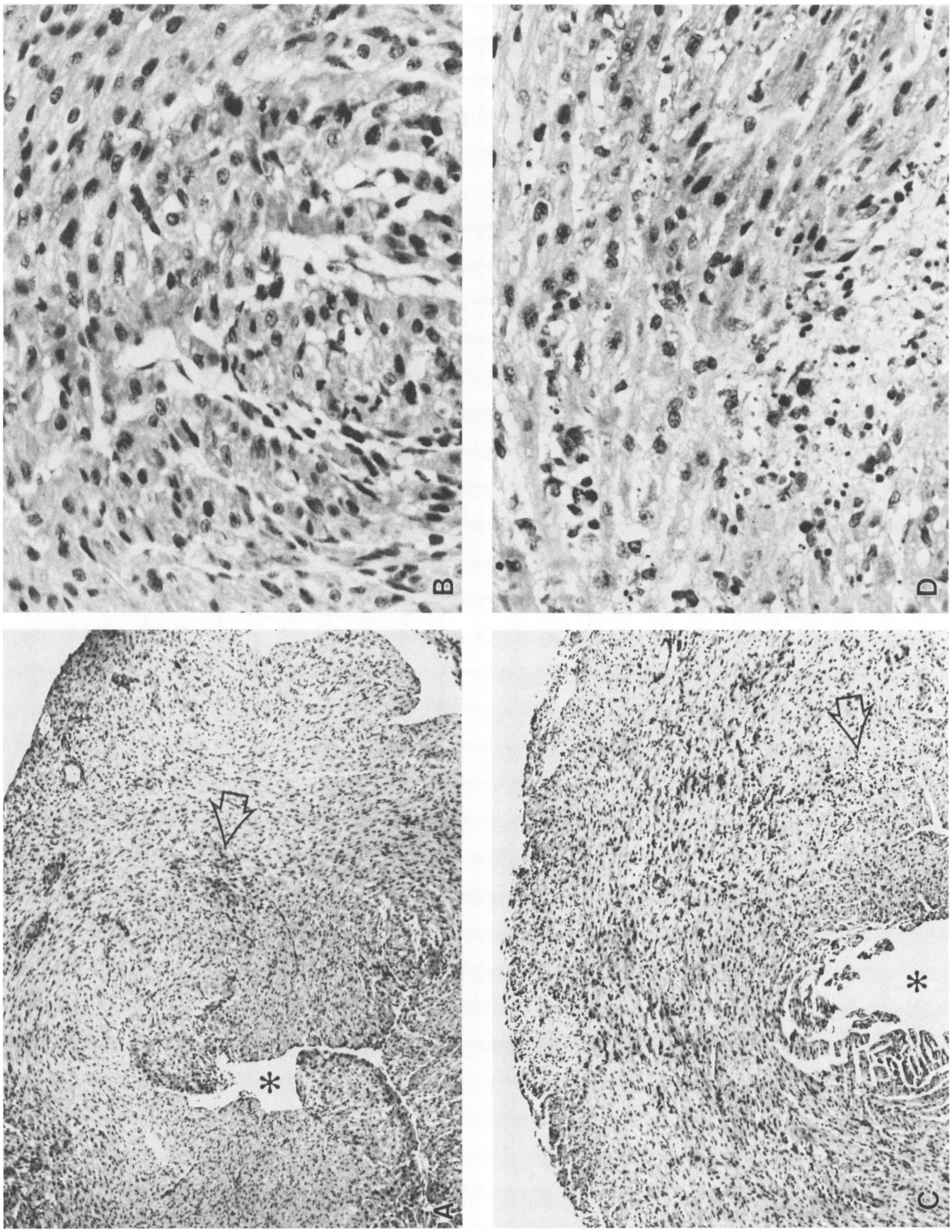
Lesions induced by T1L and T3D at 7 days postinjection, when present, resembled those of 8B at 5 days postinjection, with both polymorphonuclear and mononuclear inflammation (Fig. 2G and H). However, they occupied only a small volume overall (see below), were randomly distributed in the right and left ventricular myocardium, and were approximately 125 μm or less in diameter.

In order to quantify the difference in necrosis induced by the three viruses, we selected midheart sections and scored them blindly by using the intercept method (Table 2). This method determines the total fraction of heart tissue involved in lesions and thus takes into account both lesion size and frequency. Necrosis detected in 8B-infected hearts occurred at a frequency significantly different from that in T1L- and T3D-infected hearts as early as 3 days postinjection ($P < 0.05$). By 7 days postinjection, we detected microscopic lesions in T1L- and T3D-infected hearts, but the fraction of necrotic tissue was only 3 to 10% of that found in 8B-infected hearts. Thus, 8B induced significantly greater cardiac necrosis than did either parent, and this was evident both macroscopically and microscopically.

Electron microscopy of hearts from 8B-infected mice. We removed hearts from mice 5 or 7 days postinjection with 10^6 PFU of 8B and examined sections by electron microscopy. The earliest involvement, noted at the periphery of advanced lesions, consisted of viral arrays in apparently healthy cardiac myocytes (Fig. 3A). The presence of virus was the only abnormality noted in such cells. At the centers of advanced lesions there were obviously necrotic cells, as noted by extensive cell and/or organellar swelling, marked amorphous mitochondrial densities, and generalized structural disruption, including discontinuities in the cell membrane (Fig. 3B). Viral arrays were noted in these dead and dying myocytes. In advanced lesions, there was also considerable dystrophic calcification in necrotic cells, particularly associated with grossly abnormal mitochondria (Fig. 3C). Viral arrays were also noted in cells with dystrophic calcification.

Growth of 8B, T1L, and T3D in the heart. One possible explanation for the increased myocardial necrosis in 8B-infected animals was increased viral replication in the heart relative to that in T1L- and T3D-infected animals. To determine whether this was the case, we removed hearts from mice 1 to 9 days postinjection with approximately 10^4 PFU of 8B, T1L, or T3D and determined their viral titers (Fig. 4). Whereas T3D replicated to titers 1,000- to 10,000-fold lower than 8B on days 1 to 7 postinjection, T1L grew nearly as well as 8B during this time. Results at 9 days postinjection indicated that T1L and T3D titers were decreasing by this time. Note, however, that the key comparisons of induction of macroscopic and microscopic lesions were made on days 3 to 7 postinjection (Fig. 1 and 2, Tables 1 and 2). Note also that 8B-induced lesions increased concomitantly with increasing viral titers in the heart.

FIG. 2. Hearts from 8B-infected mice with progressively larger and more frequent microscopic lesions over time. Neonatal NIH/Sw mice were injected as described for Fig. 1, and hearts were removed on the day indicated. Sections were from 8B-infected animals on days 3 (A and B), 5 (C and D), and 7 (E and F) postinjection and from T1L (G)- and T3D (H)-infected animals on day 7 postinjection. Panels A, C, and E are at ×80 magnification; all others are at ×200 magnification. In each of the panels A, C, and E the epicardial surface is at the top, an asterisk marks the left ventricular chamber, and an arrow identifies one example of a lesion. The sections from the 8B-infected animals were typical; those from the T1L- and T3D-infected animals were selected in particular because they showed pathology (see Table 2). FIG. 2E through H continues on the following page.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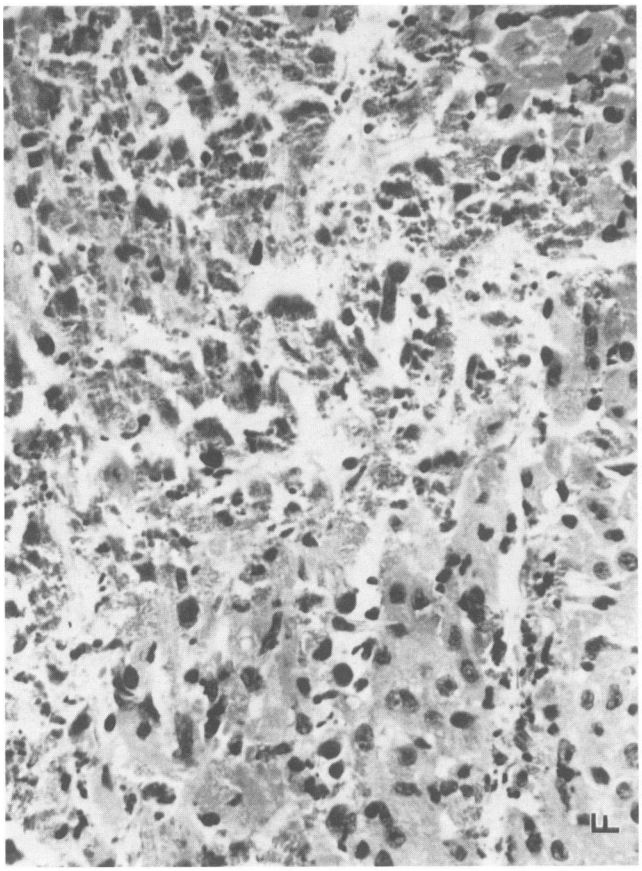
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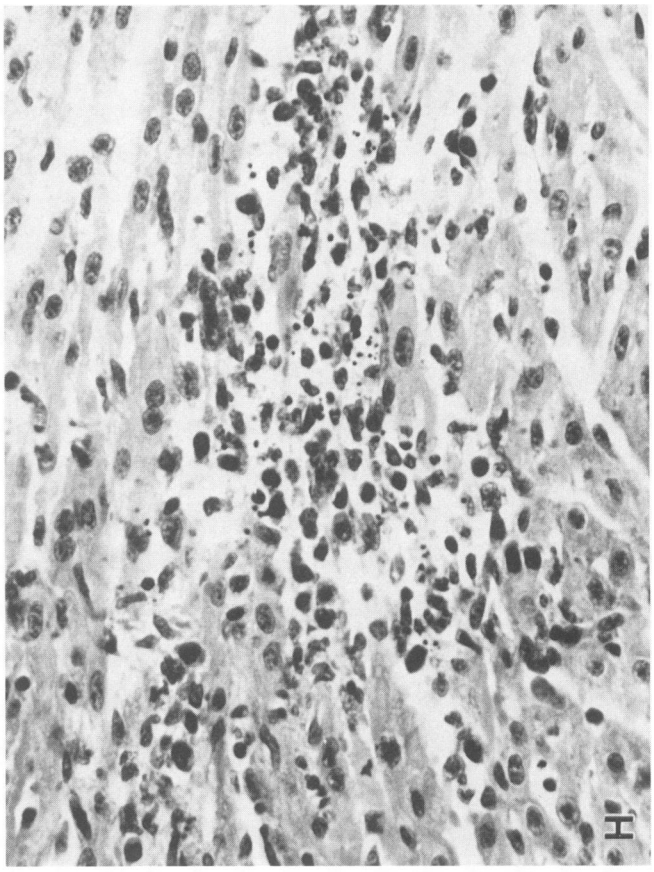
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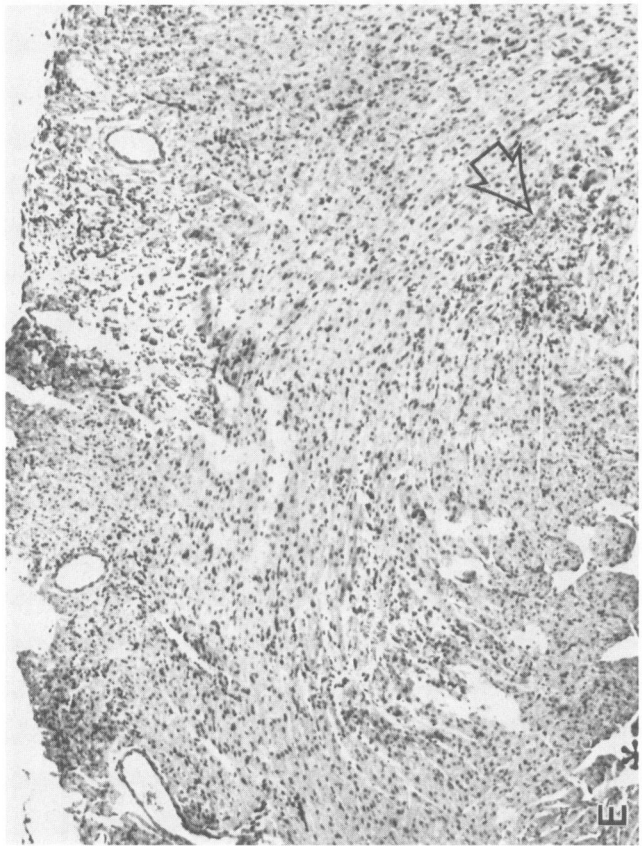
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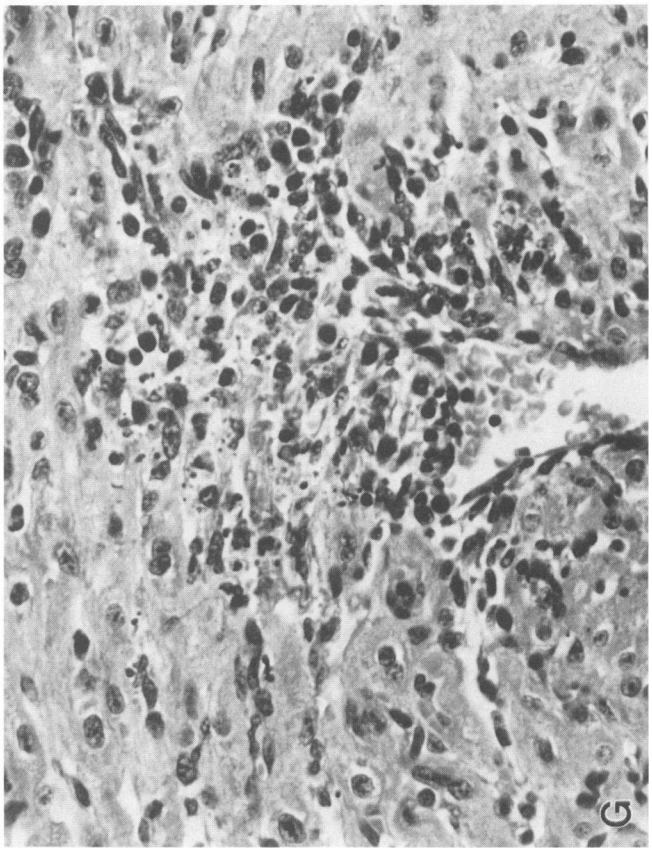
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TABLE 2. Percent necrotic myocardium in infected mice

Injection	% Necrotic myocardium on postinjection day:		
	3	5	7
Mock	0.00	0.00	0.00
T3D	0.00	0.02	0.87
T1L	0.00	0.30[a]	2.44[a]
8B	0.65[b]	8.5[c]	25.8[d]

[a] Not significantly different from T3D ($P > 0.05$).
[b] Significantly different from both T1L and T3D ($P < 0.05$).
[c] Significantly different from both T1L and T3D ($P < 0.025$).
[d] Significantly different from both T1L and T3D ($P < 0.0005$).

Although the average 8B titers in the heart were always higher than those for T1L, the ranges of individual titers in the heart were overlapping. The appearance (presence or absence of external lesions) and actual titers in the 8B- and T1L-infected hearts at 7 days postinjection are indicated in Table 3. External cardiac lesions were present in 8B-infected animals but absent in T1L-infected animals exhibiting identical or greater viral titers in the heart. The data therefore indicate that increased viral replication is not the predominant determinant of the 8B-induced myocardial necrosis.

Induction of myocardial lesions in athymic mice. Certain coxsackievirus strains which are myocarditic in BALB/c mice are nonmyocarditic in athymic (nude) BALB/c mice, suggesting that cytotoxic T cells are critical to the coxsackievirus-induced myocarditis (see Discussion). Athymic (*nu/nu*) and euthymic (+/+ or *nu*/+) neonatal BALB/c mice (nude NIH/Sw mice were unavailable) were injected with 10^6 PFU of T1L, T3D, or 8B. The animals died or were sacrificed between 5 and 14 days postinjection, and their hearts were examined for the presence of macroscopic external lesions (Table 4). Not surprisingly, T3D failed to induce lesions in euthymic or athymic mice. However, T1L induced lesions in 56% of the BALB/c euthymic mice, in contrast to its behavior in NIH/Sw mice (Table 1), indicating the influence of mouse genotype on disease outcome. 8B was efficiently myocarditic in both athymic and euthymic mice, indicating that T cells were not critical to the 8B-induced myocarditis in BALB/c mice. Since T cells were apparently also not critical to the T1L-induced myocarditis in BALB/c mice, this may be generalizable to all reovirus-induced myocarditis in BALB/c mice.

Cytopathic effect in cultured mouse L cells. Routine preparation of laboratory stocks of virus suggested that 8B was highly cytopathic to cultured mouse L cells, and this was quantified as follows. Mouse L cells were infected or mock infected at a multiplicity of infection of either 50 to 100 PFU per cell or 101 to 140 PFU per cell and then were examined for viability by trypan blue exclusion 22 h postinfection (Table 5). Although T1L and T3D did decrease the viability of the cell population by approximately 25%, 8B decreased the viability by greater than 50% and was therefore twice as cytopathic as either parent. By analogy, cytopathogenicity in vivo may also be due to a direct effect of 8B on the cells.

DISCUSSION

8B is an efficiently myocarditic reovirus variant. The reovirus variant 8B was generated by reassortment between T1L and T3D in a neonatal mouse (34). We report here that 8B, introduced into neonatal NIH/Sw mice by any of a variety of routes, efficiently induced myocarditis with accompanying gross external cardiac lesions. In contrast, the two parent viruses, T1L and T3D, induced lesions rarely or not at all in these mice. This is the first report of the generation of a myocarditic strain of reovirus from two nonmyocarditic strains and allows the first comparison between such strains.

The efficiency with which 8B induces myocarditis in neonatal mice is not unprecedented for reoviruses. T1 strain 716 is efficiently myocarditic in white Swiss Webster neonatal mice (10), and T1L is myocarditic in Prince Henry neonatal mice (33). Although our T1L strain was poorly myocarditic in NIH/Sw mice, it induced lesions in more than half of the euthymic BALB/c mice tested (albeit at a high dose of virus). Moreover, T1L-induced lesions in BALB/c mice were as frequent between 6 and 8 days postinjection as they were at 14 days postinjection (data not shown), in contrast to their increasing frequency over time in NIH/Sw mice. The mechanism by which T1L induces lesions efficiently in BALB/c mice (and apparently in Prince Henry mice [see below] but poorly in NIH/Sw mice) remains to be determined. An influence of the host mouse strain on myocarditis has also been observed with coxsackievirus (7, 35).

T3D is myocarditic in NMRI neonatal mice (6, 30); however, there is no indication in those reports as to the frequency of induction of lesions. Our T3D strain was essentially nonmyocarditic, potentially reflecting an influence of the host mouse strain, as mentioned above. However, Goller et al. (6) passaged their T3D strains repeatedly in tissue culture (14 times), which is likely to have resulted in the accumulation of mutations in their virus stock since reoviruses mutate at a high frequency characteristic of RNA viruses in general (11, 29). These mutations could have altered the behavior of the virus in vivo. Stangl et al. (30) did not report the passage history of their T3D strain; however, their inocula contained 10 μg of virus each (presumably corresponding to 10^9 PFU), and we have not tested such high doses. Finally, it is worth noting that the T1L strain of Walters et al. (33) was also passaged extensively (19 to 23 times in culture). The difficulty in comparing the results from studies using virus strains with different (and extensive) passage histories emphasizes the usefulness of a comparison between a myocarditic variant and related nonmyocarditic strains with similar, limited passage in tissue culture.

The greater potential for our T1L strain to induce cardiac lesions than for our T3D strain to do so is probably an important factor in the efficiently myocarditic phenotype of 8B. Genetic analysis has revealed that the 8B gene associated with efficient induction of cardiac lesions was derived from the T1L parent (the M1 gene) (28).

Viral replication in the heart is not the predominant determinant of myocarditis. Although T3D replicated poorly in the hearts of infected mice, T1L grew nearly as well as 8B. At 5 and 7 days postinjection, when gross external cardiac lesions were apparent in most 8B-infected mice but in no T1L-infected mice, 8B had achieved average titers only three to four times those seen for T1L. Moreover, gross external cardiac lesions were present on 8B-infected hearts but were absent on T1L-infected hearts exhibiting identical or greater viral titers. In addition, several progeny reassortant viruses derived from 8B which exhibit the myocarditic phenotype achieved titers only equal to or lower than those for T1L (28). Finally, it is worth noting that although T1L grew to titers 1,000- to 10,000-fold higher than T3D, there was no significant difference ($P > 0.05$ [Table 2]) in the degree of myocardial necrosis induced by the two viruses between 3 and 7 days postinjection (although T1L did occasionally induce external lesions, whereas T3D never did so [Table

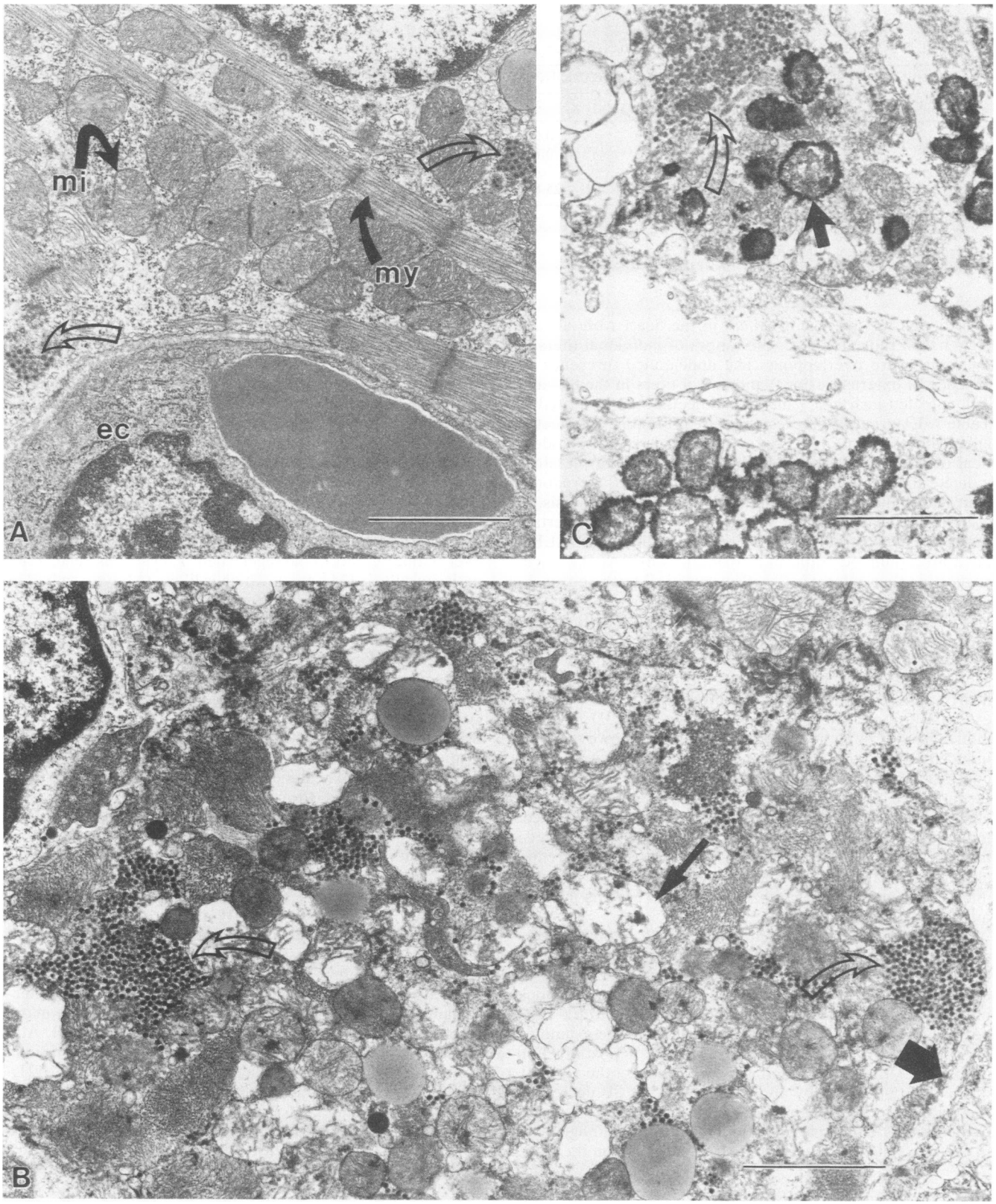

FIG. 3. Electron micrographs of hearts from 8B-infected mice. Neonatal NIH/Sw mice were injected with 8B as described for Fig. 1, and hearts were removed at 5 or 7 days postinjection. In all panels, viral arrays are indicated by open arrows. (A) Infected but completely viable cardiac myocyte. Normal-appearing mitochondria (mi) and myofibrils (my) are indicated. An adjacent capillary with its morphologically normal endothelial cell (ec) and lack of virus is also indicated. (B) Necrotic cardiac myocyte demonstrating severe architectural disruption, including swollen and fractured mitochondria with numerous amorphous densities (thin arrow) and markedly damaged cell membrane (broad arrow). (C) Dystrophic calcification in an advanced lesion demonstrating mineral predominantly associated with mitochondria (arrow) in severely disrupted, necrotic cells. All magnifications were originally ×12,500. Bar, 2 μm.

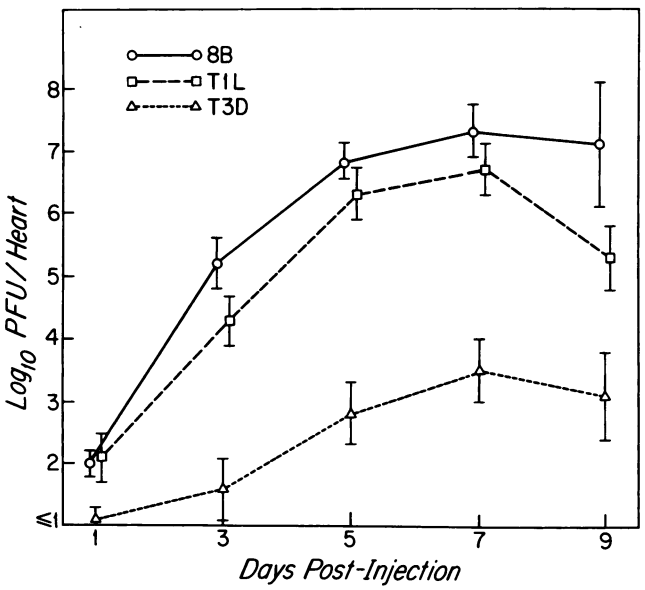

FIG. 4. Growth of virus in the hearts of infected mice. Neonatal NIH/Sw mice were injected intramuscularly in the left hindlimb with 10^4 PFU of 8B, T1L, or T3D. Hearts were removed on the day indicated, and the viral titers were determined by plaque assay. Datum points on day 1 represent the average of 2 mice each, and that for the 8B titer on day 9 was an average of 3 mice; all other datum points represent the averages of a minimum of 7 mice each, with most using 12 mice each. Error bars represent two standard errors of the mean (95% confidence level).

1]). The results suggest that viral replication is not the predominant determinant of the 8B myocarditic phenotype. Studies of myocarditic and nonmyocarditic strains of coxsackievirus have failed to document a clear difference in the titers achieved in the heart, although several authors have suggested there might be a general trend relating viral growth and myocarditis (3–5).

TABLE 3. Viral titer and external lesion incidence in hearts

Virus injected	Titer[a]	Lesions[b]
8B	5.6	−
	6.7	+
	6.9	−
	7.0	+
	7.3	+
	7.4	+
	7.4	−
	7.6	+
	7.7	−
	7.7	+
	7.8	+
	7.9	+
T1L	5.7	−
	5.7	−
	6.2	−
	6.5	−
	6.7	−
	6.7	−
	6.7	−
	6.8	−
	7.0	−
	7.3	−
	7.5	−
	7.8	−

[a] Log PFU in individual hearts at 7 days postinjection with 10^4 PFU.
[b] Presence of even one external lesion is indicated (+).

TABLE 4. Induction of external cardiac lesions in athymic mice

Virus[a]	Mouse type (BALB/c)	% of mice with cardiac lesions[b] (no. of positive hearts/total)
T3D	+/+ or *nu*/+	0 (0/19)
T3D	*nu*/*nu*	0 (0/4)
T1L	+/+ or *nu*/+	56 (15/27)
T1L	*nu*/*nu*	50 (4/8)
8B	+/+ or *nu*/+	100 (19/19)
8B	*nu*/*nu*	100 (7/7)

[a] Mice were injected with 10^6 PFU of virus and died or were sacrificed between 5 and 14 days postinjection. The fraction of positive hearts did not vary appreciably over this time period.
[b] Positive hearts had one or more external lesions.

The role of the immune system in viral myocarditis. Neonatal athymic (nude) or euthymic BALB/c mice were injected with 8B in order to determine whether T cells were required for generation of myocarditis. 8B induced macroscopic external cardiac lesions in all euthymic and athymic mice, indicating that T cells were not required. Examination of several histological sections from the athymic mice 7 days postinjection revealed an infiltrate with an equivalent number of polymorphonuclear leukocytes but fewer mononuclear cells than seen in euthymic mice (data not shown).

The question of T-cell involvement in coxsackievirus-induced myocarditis has been controversial for the past decade, although much of the controversy can be resolved as differences in mouse or virus strains (for a review, see reference 15). One body of data suggests that T cells are essential for, and potentially the predominant mediators of, coxsackievirus-induced myocarditis. Peak viral titers in the heart precede peak necrosis in coxsackievirus B3 (CVB3)-infected BALB/c and C3H/HeJ mice (7, 23), suggesting that necrosis is not a direct result of viral replication. Note that this result contrasts with our results for reovirus 8B-induced myocarditis. In addition, CVB3-inoculated thymectomized or athymic BALB/c mice do not develop lesions; however, if they are reconstituted with immune spleen cells (9, 37), particularly cytolytic T cells (8), they do develop lesions. Finally, a differential cytolytic T-cell response remains one of the few proposed differences between myocarditic and nonmyocarditic coxsackievirus strains (12).

A second body of data, however, suggests that T cells are not required for the coxsackievirus-induced myocarditis, in that cardiac lesions are found in infected athymic as well as euthymic mice (22, 24, 25). In addition, cytolytic T-cell activity is undetectable in CVB3-infected BALB/c mice with myocarditis (7), and CVB4 induces myocarditis in ICR Swiss

TABLE 5. Cytopathic effect in cultured mouse L cells

Virus	% Viable mouse L cells[a] at a multiplicity of infection of:	
	50 to 100	101 to 140
None (mock)	100	100
T3D	73	77
T1L	78	70
8B	46[b]	42[b]

[a] Determined by trypan blue exclusion; results were normalized to mock-infected samples.
[b] Significantly different from T1L and T3D ($P < 0.03$).

mice depleted of T cells by antithymocyte serum (14). These data are more consistent with our results for reovirus 8B-induced myocarditis.

Natural killer cells (present in athymic mice) have been implicated as mediators of cardiac damage in CVB3-infected female BALB/c mice (13). In addition, several studies have suggested that CVB3-induced myocardial necrosis can induce autoantibodies to cardiac myosin in susceptible mouse strains, leading to chronic myocarditis (18, 19, 35). The 8B-induced lesions occur at early times postinjection, suggesting that the 8B-induced myocarditis is not antibody mediated. Moreover, 8B is cytopathic to cultured cells, suggesting that the myocardial necrosis is probably a direct viral effect (see below).

A potential mechanism for the 8B-induced myocarditis. The data suggest that the myocardial necrosis seen in 8B-infected mice is a direct effect of viral injury to cardiac myocytes. That is, viral arrays were present in necrotic myocytes in infected animals, and the inflammatory response noted by light microscopy in advanced lesions was most consistent with an inflammatory response to myocytes which were already necrotic rather than a direct inflammatory cell-mediated injury. Moreover, 8B induced peak myocardial necrosis coincident with peak viral titers in the hearts of infected mice and caused myocarditis in athymic mice. Finally, 8B was more cytopathic than either T1L or T3D in cultured mouse L cells, and this was also true in primary cultures of mouse macrophages (Herbert Virgin, personal communication) and mouse cardiac myocytes (Yoshiki Matoba, personal communication).

Specific reovirus genes have been implicated in the inhibition of host cell DNA, RNA, and protein synthesis (26, 27). It is possible that 8B affects one of these host cell functions in a way different than that of the parent viruses, either directly or indirectly, thereby compromising the health of the cell. Reassortant viruses derived from 8B can be used to determine which gene(s) is associated with its myocarditic phenotype (28) in order to address this question.

8B, like many reoviruses, is pantropic in neonatal mice. It grows to high titers in the liver and brain and potentially in other organs as well (data not shown). The observed cytopathicity in a number of different cell types infected with 8B in culture suggests that this virus has the potential to be highly cytopathic to any cell type which is permissive for its replication. Infection with 8B is fatal to the neonatal mouse, and the degree of myocardial necrosis at the time of death is consistent with myocarditis as the cause of death, although other organ failure could also play a role. Further studies, including a genetic analysis using reovirus reassortants, will be directed towards a better understanding of the mechanism(s) of 8B-induced myocarditis and pathogenesis in neonatal mice.

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