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Assessment of Coxsackievirus B3 *ts* Mutants for Induction of Myocarditis in a Murine Model

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Ten temperature-sensitive (*ts*) mutants isolated from a myocarditis-inducing wild-type (WT) coxsackievirus B3 parent did not induce myocarditis in adolescent CD-1 mice. An avirulent prototype *ts* mutant from one of the three complementation groups adsorbed to murine cardiac tissue, as did WT virus. Heart tissues from mice inoculated with WT virus contained 100- to 1,000-fold more virus than heart tissues from mice inoculated with any of the three prototype *ts* mutants. WT virus exhibited a greater capsid stability and a higher efficiency of replication at 37°C than any of the three prototype *ts* mutants. All three prototype *ts* mutants induced less interferon in vivo than WT virus. Cell-mediated immune responses, assessed by the cell migration inhibition assay, were different in mice inoculated with WT virus when compared to *ts* 5 mutant virus. Peritoneal exudate cells from mice inoculated with WT but not *ts* 5 virus reacted specifically against antigens in WT virus HeLa cell lysates and antigens extracted with KCl from cardiac tissues of mice inoculated with WT virus. Cardiac tissues of mice inoculated with WT but not *ts* 5 virus contained KCl-extractable antigens which were able to specifically inhibit the migration of peritoneal exudate cells taken from mice immunized with WT virus. Therefore, *ts* 5 neither elicited a measurable cell-mediated immune response nor induced antigens in cardiac tissues which were immunoreactive with sensitized-(WT virus)-peritoneal exudate cells. Of 9 revertant viruses isolated from the 10 *ts* mutants, 5 showed covariance in ability to replicate at 39.5°C and capacity for induction of myocarditis. Some revertants exhibited a reduced capsid thermostability compared to WT virus but yet retained the capacity for induction of myocarditis. The data suggest that induction of myocarditis by coxsackievirus B3 variants depends on a combination of several variables, including capsid stability, capacity for replication at 37°C, and expression of the three identified genes. All three prototype *ts* mutants served as vaccine viruses in preventing myocarditis in adolescent mice subsequently challenged with WT virus. However, all three prototype *ts* mutants and their revertant variants retained partial to complete lethality in CD-1 neonates.

In recent years, many different viruses have been associated with human heart diseases (3). Of the many viruses involved, more evidence has been accumulated which implicates coxsackievirus B1-B5 with serious and frequently fatal cardiac involvement in both neonates and adults (10, 12, 13). A murine model has been used by several investigators to study coxsackievirus group B-induced heart disease. Histologically, the mouse myocardial lesions resemble lesions found in the human myocardium after coxsackievirus group B infections (3, 22, 25).

Currently, there is evidence which suggests at least two different mechanisms for the development of heart disease after experimental coxsackievirus group B infection in the murine model. Woodruff and Woodruff (27) have demonstrated the involvement of T lymphocytes in the pathogenesis of coxsackievirus B3 heart disease. They reported that severity of myocarditis is significantly reduced in T cell-deprived, coxsackievirus B3-infected mice and suggested that T cell-mediated reactions might be involved in the destruction of myocytes. In contrast, Rager-Zisman and Allison (20) reported that cardiac damage after coxsackievirus B3 inoculation was more severe in immunosuppressed than in normal mice. These investigators observed higher infectious virus titers and more severe myocardial lesions in infected cyclophosphamide-treated mice than in controls.

Conditional-lethal mutants provide a unique opportunity to examine the relationships between genetic properties (e.g., ability to induce

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myocarditis) and a specific genetic defect. Some temperature-sensitive (*ts*) viruses are frequently less pathogenic than the parental strain from which they are derived. The reason for this apparent correlation between a *ts* defect and avirulence is unkown. The present paper compares murine host responses to the myocarditic wild-type (WT) parental virus and amyocarditic *ts* mutants. Several properties of these viruses, which may contribute to pathogenicity, are described. Isolation of the coxsackievirus B3 *ts* mutants and assignment to three complementation groups were described previously (24). Revertant variants of the three prototype *ts* mutants were also isolated and examined for pathogenicity.

MATERIALS AND METHODS

Cell culture and media. HeLa cells used in this study were obtained from the American Type Culture Collection, and stocks were grown in Auto-Pow minimum essential medium (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 μg of neomycin sulfate per ml, 2.5 μg of amphotericin B per ml, and 0.056% $NaHCO_3$. All other cell culture reagents were purchased from Grand Island Biological Co., Grand Island, N.Y. L929 cells were obtained from W. Magee, University of Texas at San Antonio, and propagated in the same medium used for HeLa cells.

Virus stocks and plaque assay system. Coxsackievirus B3, obtained from J. F. Woodruff, Cornell University Medical College, New York, N.Y., was plaque purified, and plaque assays were performed in HeLa cells as previously described (24). A *ts* mutant from each of three complementation groups was employed as a prototype representative (e.g., *ts* 1, *ts* 5 and *ts* 11). Based on data to be submitted elsewhere for publication, the defective lesion associated with these *ts* viruses appears to be associated with polypeptide processing, capsid assembly, and ribonucleic acid synthesis for *ts* 1, *ts* 5, and *ts* 11, respectively. The precision of the plaque assay procedure has been calculated to be ±22% experimental error at a 95% confidence level, similar to an earlier report (2). Photosensitized virus stocks were propagated in HeLa cells in the presence of 4 μg of neutral red per ml as previously described (11). Photosensitized virus was inactivated by irradiation with a 375-W photoflood lamp (36.8 mW/cm^2) for 200 s as previously described (24). Revertants of *ts* mutants were isolated from plaques formed in HeLa cells at 39.5°C and plaque purified. Virus stocks were prepared in HeLa cells at 34°C and then assayed at 39.5 and 34°C. Virus particles were purified as described previously (7). [5-^{3}H]uridine was obtained from Schwarz/Mann Chemicals (Orangeburg, N.Y.) for labeling viral ribonucleic acid. Virus-inoculated cells which were incubated at 34°C were pulsed with 5 μCi/ml at 4.5 to 5.5 h post-inoculation (p.i.). Cells incubated at 39.5°C were routinely pulsed with [^{3}H]uridine 1 h earlier. Adsorption of WT and *ts* 11 to mouse heart tissue was compared by mixing constant amounts of purified [^{3}H]uridine-labeled virus with aliquots of homogenized hearts. Aliquots of virus-challenged heart tissues were rinsed three times with phosphate-buffered saline at 0, 10, 30, 60, and 120 min after virus challenge, and tissue-associated trichloroacetic acid-insoluble counts per minute were measured.

Mice. All in vivo experiments were performed with adolescent CD-1 mice obtained from Charles River, Inc., Wilmington, Mass. Newborn mice were defined as mice less than 24 h of age, and adolescent mice were approximately 4 to 6 weeks old. Adolescent mice were inoculated via the intraperitoneal (i.p.) route with 0.1 ml containing approximately 10^7 plaque-forming units (PFU). Newborn mice were inoculated by intracerebral, subcutaneous, i.p., or a combination of these routes with 0.03 to 0.05 ml of a stock virus solution containing 10^7 PFU/ml.

Histology. Hearts were removed and fixed in 10% Formalin (diluted in phosphate-buffered saline, pH 7.4), embedded in paraffin, and processed for histological examination (6). Sections of each heart were stained with hematoxylin and eosin. A minimum of two to four sections per mouse heart were examined for myocardial lesions at 100× with an Olympus KHC light microscope. The presence of at least one lesion in each of two sections from a single heart was required for describing a heart as positive for myocarditis.

MIF assay. The agarose droplet cell migration inhibition factor (MIF) assay, as described by Harrington and Stastny (9), was employed as an in vitro correlate of cellular immunity. Coxsackievirus B3-sensitized mouse peritoneal exudate cells (MPEC) were prepared as follows. Adolescent mice were immunized i.p. with 10^7 PFU in 0.1 ml of Hanks balanced salt solution, and 2 weeks later the mice were boosted by i.p. injection with 0.1 ml of 10^8 PFU of virus. After 2 weeks, the mice were given an i.p. injection of 2 ml of light mineral oil, and 3 days later the MPEC were collected in sterile Hanks balanced salt solution. The cells were washed three times in Hanks balanced salt solution and immediately incorporated into the agarose droplet MIF assay (9). Heart antigens used in the latter assay were obtained from 35 to 40 hearts of mice sacrificed at 8 days p.i. with WT virus. The cardiac tissue was extracted with hypertonic KCl by the methods of Reisfeld and Kahan (21) with modifications of Meltzer et al. (17), except that cardiac tissues were disrupted by three 1-min shearings at 0°C with a Lourdes Omnimixer at maximum setting. In calculation of the MIF results, a mean migration index of ≤65% was considered a conservative estimate for assessing positive inhibition of macrophage migration (1).

Interferon assay. Adolescent CD-1 mice were inoculated i.p. with 10^7 PFU of each virus per 0.2 ml. At 24 or 48 h p.i., groups of 5 mice each were sacrificed, and blood was removed. Serum was adjusted to pH 2 and held for 18 h at 4°C, after which the pH was readjusted to pH 7.2 with sterile 1 N NaOH. Monolayer cultures of L929 cells were incubated for 24 h at 37°C with dilutions of these sera in minimal essential medium containing 10% fetal calf serum and 50 μg of gentamicin per ml (Schering Corp., New York, N.Y.). The fluids were removed, and the cells were washed twice with minimal essential medium and inoculated

with approximately 50 to 100 PFU of vesicular stomatitis virus per monolayer cell culture, and plaques were enumerated 2 days after incubation in CO_2 at 37°C as previously described (23). One unit of interferon is defined as the reciprocal of the highest dilution, which gives approximately a 50% reduction in plaque number. In this assay, 1 National Institutes of Health international reference unit of mouse interferon titered as 1 U.

RESULTS

Histopathology of the myocardium from adolescent CD-1 mice inoculated with coxsackievirus B3 *ts* mutants. Myocardial lesions in CD-1 mice are focal (rather than diffuse), irregularly shaped areas containing numerous monocytes surrounding myocytes which appeared to be undergoing degradation. A typical section of a heart obtained from a mouse inoculated with WT virus and sacrificed at 8 days p.i. is shown in Fig. 1. Numbers of myocytes involved in a discrete foci varied from a few to several hundred. A prominent feature of the typical myocardial lesion was the interstitial infiltration of mononuclear and some polymorphonuclear leukocytes.

The capability of the *ts* mutants for induction of myocarditis in adolescent CD-1 mice is summarized in Table 1. Because 8 days p.i. was found to be the optimum time for detection of myocardial lesions in adolescent mice given WT virus, this time was selected for examining mice given the *ts* mutants. None of the 10 *ts* mutants was found to induce myocardial lesions. Revertant viruses from 9 of 10 *ts* mutants were inoculated into mice as previously described (a revertant of *ts* 3 has not been isolated). Histological evidence of myocarditis was, however, obtained in mice inoculated with five of the nine revertants (Table 2). Only four revertants of *ts* mutants from complementation groups I (two of two) and III (two of five) were able to produce myocarditis.

Studies of the basis for pathogenicity of coxsackievirus B3. The amount of infectious virus present in mouse hearts after inoculation with WT and prototype *ts* mutants was assessed to determine whether there was any correlation between production of myocarditis and amount of infectious virus present. In experiment 1 of Table 3, each virus was inoculated into a group of five adolescent CD-1 mice at a concentration of 10^8 PFU per mouse. At 8 days p.i., the infectivity titers ranged from 4.9×10^3 PFU/ml for WT virus to $<1.0 \times 10^2$ PFU/ml (lower limit of detection in the assay system) for *ts* 5 and *ts* 11. In experiment 2, mice were inoculated with neutral red-tagged virus under darkroom-safelight conditions. At 3 days after inoculation, the hearts were removed and assayed for both residual photosensitized virus plus progeny virus (titrations conducted in the dark) or only progeny virus (titrations made after photoinactivation). Infectivity titers were highest for WT virus, and very little if any replication of *ts* mutants could be demonstrated in heart tissue at a time (3 days p.i.) when WT virus titers were at a maximum (Gauntt, unpublished data). Revertant viruses of each of the three prototype *ts* viruses replicated to approximately the same titer as did the WT virus (experiment 1). Because one of the three revertant viruses (*ts* 5R) does not cause myocarditis, there is no absolute correlation between amount of virus in heart tissues and presence of myocarditis.

The body temperature of a mouse is approximately 37 to 38°C, and thus the inability of a *ts* mutant to replicate at this temperature could explain the lack of pathogenicity (myocarditis). The efficiencies of plating for WT and prototype *ts* mutants of coxsackievirus B3 at 34, 37, and 39.5°C were measured and are shown in Fig. 2. WT virus formed similar numbers of plaques at the three different temperatures. However, each prototype *ts* mutant was less successful at forming plaques at the higher incubation temperatures of 37 and 39.5°C, with replication of *ts* 1 being most sensitive at these two temperatures.

Defects in capsid polypeptides of *ts* mutant viruses are commonly manifested by greater heat lability of mutant virions than the WT virus, suggesting alterations in the amino acid sequence and improper folding of capsid proteins during maturation. The heat stabilities of WT and the three prototype *ts* mutant viruses were compared at 50°C (Fig. 3). Each prototype *ts* mutant (*ts* 1, *ts* 5, and *ts* 11) was more readily heat inactivated at 50°C than was the WT virus. After approximately 2 to 3 min at 50°C, only 1% of each of the *ts* mutants remained infectious, whereas approximately 7 to 8 min at 50°C was required to heat inactivate 99% of the WT virus. These results suggest that the *ts* mutants may have defects in structural polypeptides. Revertants of the three *ts* mutants were similarly examined for temperature stability at 50°C. Whereas the revertants of *ts* 1 and *ts* 11 were as stable as the WT virus, the revertant of *ts* 5 closely resembled the prototype *ts* mutants in temperature stability (data not shown).

Adsorption of radiolabeled WT and *ts* 11 coxsackievirus B3 to CD-1 adolescent mouse heart tissue in vitro was measured to determine whether lack of attachment of a *ts* mutant could explain the lack of pathogenicity (Table 4). After 10 min, 52% of the input ^{3}H-labeled WT virus was tissue associated. A similar amount of ^{3}H-labeled *ts* 11 virus (36%) became tissue associ-

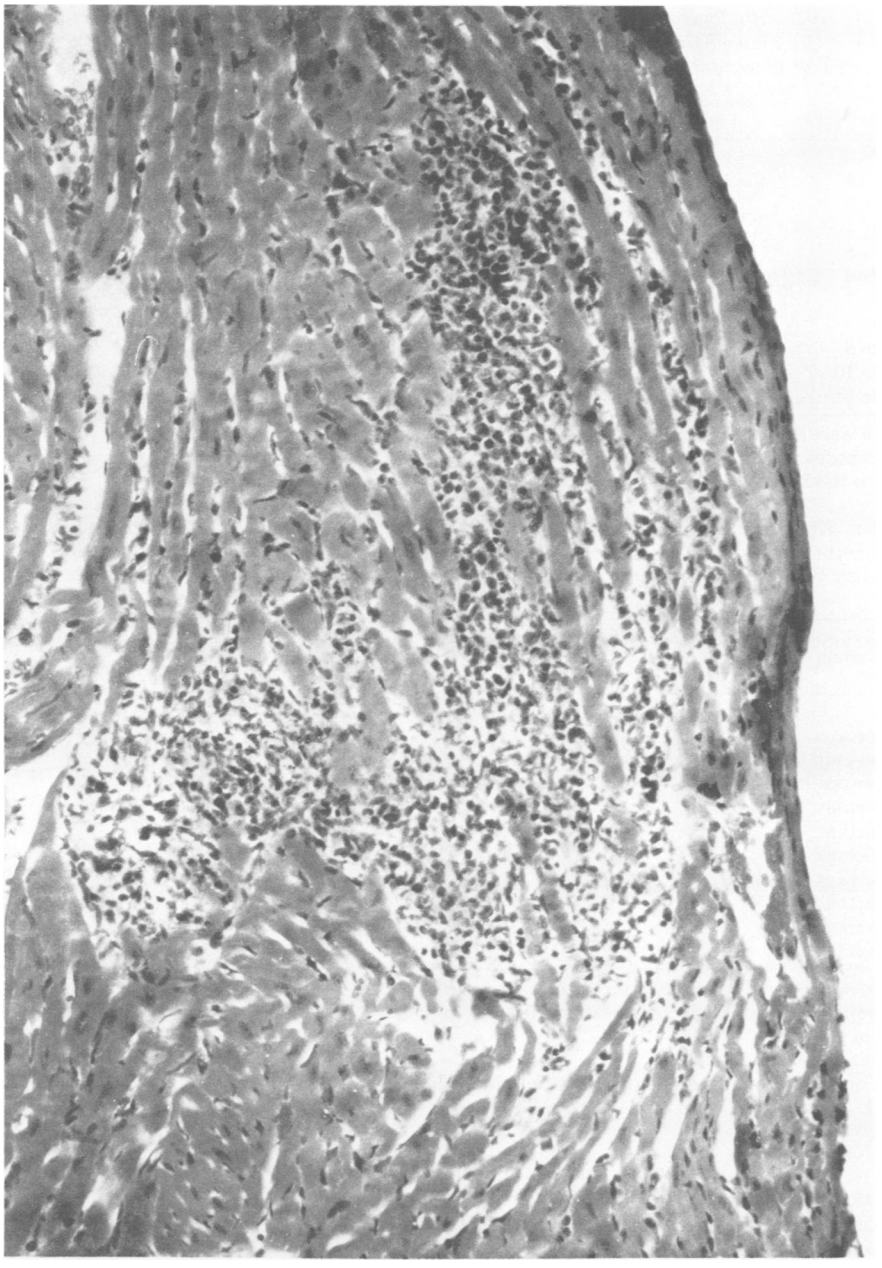

FIG. 1. *A typical myocardial lesion in cardiac tissue taken from an adolescent CD-1 mouse at 8 days p.i. with WT coxsackievirus B3. The mouse was inoculated i.p. with 10^8 PFU. Thin sections of heart were prepared and stained with hematoxylin and eosin, as described in the text. The smaller, darker-staining mononuclear cell infiltrate occupies a major portion of the thin section, whereas the lighter staining areas are normal myocytes which make up the myocardium. ×200.*

ated in 10 min, and elution of virus and degradation of the capsid also occurred thereafter. Because both viruses attached at similar levels of efficiency, differences in adsorption of these viruses did not occur and cannot explain the differences in pathogenicity. Chicken heart tissue was prepared as a nonspecific adsorption control in the same manner as the mouse heart tissue. Only 8 to 9% of the input ^{3}H-labeled WT virus was tissue associated by 30 min (data not

TABLE 1. *Histopathological studies of hearts from adolescent CD-1 mice 8 days after i.p. inoculation with WT or ts mutants of coxsackievirus B3*

Virus strain	No. of mice inoculated i.p.	Myocardial lesions[a]
WT	35	Yes
ts 1	24	No
ts 3	8	No
ts 4	8	No
ts 5	24	No
ts 6	8	No
ts 7	8	No
ts 8	8	No
ts 9	8	No
ts 10	8	No
ts 11	24	No

[a] Mice were sacrificed at 8 days p.i., and their hearts were prepared for histopathological studies as described in the text.

TABLE 2. *Histopathological studies of hearts from adolescent CD-1 mice 8 days after i.p. inoculation with WT or revertants of ts mutants of coxsackievirus B3*

Virus strain	Complementation group	No. of mice inoculated i.p.	Myocardial lesions[a]
WT		6	Yes
ts 1 revertant	III	6	Yes
ts 3 revertant[b]	II		
ts 4 revertant	I	6	Yes
ts 5 revertant	II	6	No
ts 6 revertant	III	6	No
ts 7 revertant	III	6	No
ts 8 revertant	III	6	No
ts 9 revertant[c]	—	6	Yes
ts 10 revertant	III	6	Yes
ts 11 revertant	I	6	Yes

[a] Mice were sacrificed 8 days p.i., and their hearts were prepared for histopathological studies as described in the text.

[b] Revertant not isolated.

[c] *ts* 9 does not belong to complementation group I, II, or III.

shown), demonstrating that attachment of coxsackievirus to the adolescent CD-1 mouse heart tissue was probably to specific receptor sites.

CMI responses in mice inoculated with *ts* mutants. It was of interest to determine whether mice inoculated with WT or *ts* mutants exhibited similar qualitative degrees of cell-mediated immunity (CMI) toward virion antigens and immunoreactive antigens extracted with KCl from hearts of mice at 8 days p.i. with WT virus. We have previously shown (18) that mice inoculated with WT virus ($CVB3_m$) contain cardiac antigens which, upon extraction with hypertonic KCl, specifically inhibit the migration

TABLE 3. *Measurement of infectious virus present in CD-1 adolescent mouse hearts after inoculation with WT, prototype ts mutants, or ts mutant revertants*

Expt	Virus[a]	Harvest time p.i. (days)	Virus titer in heart tissue (PFU/ml)[b]
1	WT	8	4.9×10^3
	ts 1	8	1.7×10^3
	ts 5	8	$<1.0 \times 10^2$
	ts 11	8	$<1.0 \times 10^2$
2[c]	WT	3	4.3×10^5
			3.4×10^5
	ts 1	3	1.0×10^2
			3.0×10^2
	ts 5	3	$<1.0 \times 10^2$
			$<1.0 \times 10^2$
	ts 11	3	$<1.0 \times 10^2$
			$<1.0 \times 10^2$

[a] Inoculum of 10^8 PFU per mouse.

[b] Heart tissue in a 10% suspension.

[c] Employed neutral red-sensitized virus which was inoculated i.p. under darkroom-safelight conditions as described in the text. First titer value was determined under safelight conditions (i.e., residual inoculum and progeny virus), and second titer value was determined after photoinactivation (i.e., progeny virus only).

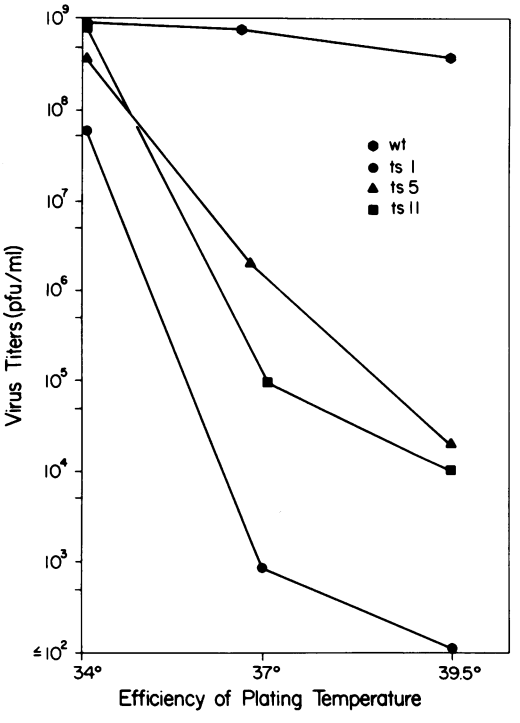

FIG. 2. *Efficiency of plating of WT and prototype ts mutant viruses at 34, 37, and 39.5°C. Aliquots of stock viruses were assayed in HeLa cells incubated at the indicated temperatures.*

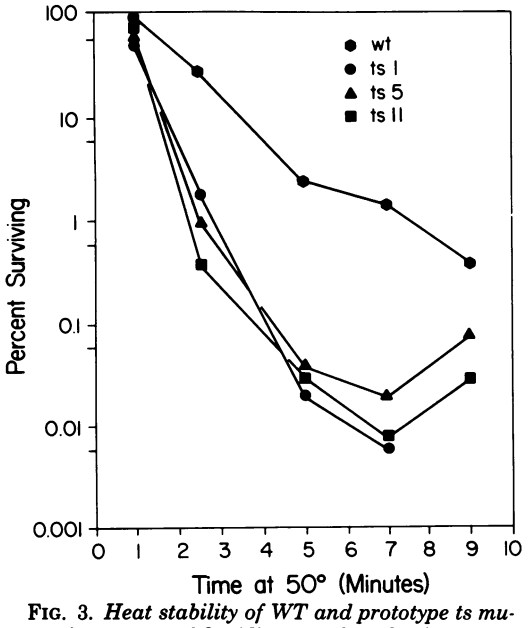

FIG. 3. *Heat stability of WT and prototype ts mutant viruses at 50°C. Aliquots of each virus were placed at 50°C and at intervals aliquots of each virus were removed, chilled to 0°C, and assayed by the plaque method as described in the text.*

of PEC obtained from mice sensitized to WT virus. The MIF assay was used to assess this host response. MPEC were obtained from adolescent CD-1 mice inoculated with WT or *ts* 5 viruses. The specific inhibition of migration of MPEC from WT- but not *ts* 5-inoculated mice with soluble antigens contained in KCl extracts of normal cardiac tissues and cardiac tissues taken from inoculated mice as well as uninfected HeLa cell and WT virus lysates from HeLa cells is demonstrated in Table 5. Concentrations of 280 and 140 μg of proteins per 0.1 ml in KCl heart extracts from WT virus-inoculated mice specifically inhibited the migration of immune PEC harvested from mice inoculated with WT virus but not *ts* 5 virus. As a control, a concentration of 280 μg of KCl heart extract from normal mouse heart tissues per 0.1 ml failed to inhibit migration of PEC from mice inoculated with either WT or *ts* 5 viruses. MPEC from mice inoculated with WT but not *ts* 5 virus also reacted with antigens in a WT virus lysate containing 10^8 and 10^7 PFU. We have previously shown that MPEC from mice inoculated with WT virus reacted with antigens contained in 10^8 PFU of purified WT virions but not at 10^7 PFU of purified virions (18). MPEC from mice inoculated with either virus did not react with antigens in uninfected HeLa cell lysates at protein concentrations similar to the levels in HeLa cell lysates containing 10^8 PFU/0.1 ml.

Our next experiments assessed whether cardiac tissue from mice inoculated with *ts* 5 and harvested at 8 days p.i. contained KCl-extractable antigens which would exhibit immunoreactivity in the MIF assay similar to KCl-extracted antigens from WT virus-inoculated mice. The results are shown in Table 6. In two experiments, KCl-extracted cardiac tissue antigens from WT- but not *ts* 5-inoculated mice inhibited the migration of MPEC from mice immunized with WT virus. Thus, *ts* 5 is unable to induce antigenic changes in cardiac tissues which are detectable by this technique in cardiac tissues from mice inoculated with WT virus.

Induction of interferon in vivo by *ts* mutants. The ability of WT and *ts* coxsackievirus B3 to induce interferon in adolescent CD-1 mice was assessed at 24 and 48 h p.i. (Table 7). Interferon titers were not measurable at 24 h (i.e., <10), but at 48 h p.i. the mice inoculated with WT virus had serum interferon titers of 40. Thus, the avirulence property of the prototype *ts* mutants (inability to cause myocarditis) does not seem to be related to the induction of interferon, because only the virulent WT virus displayed a limited ability to induce interferon.

Capacity of *ts* mutants to serve as vaccine strains in protection against myocarditis. The *ts* mutants exhibit little capacity for replication in cardiac tissues, for induction of interferon or for induction of measurable CMI. Thus, they appeared to be good candidates for vaccine strains because of their relatively inert biological activities. Groups of 20 mice were inoculated i.p. with 10^6 PFU of WT, *ts* 1, *ts* 5, or

TABLE 4. *Adsorption of purified ^{3}H-labeled WT or ts 11 mutant viruses to homogenized adolescent CD-1 mouse heart tissues*

Virus[a]	Time (min p.i.)	Tissue-associated cpm[b]	% Input cpm bound
WT	0[c]		
	10	4,625	52.0
	30	2,325	26.0
	60	1,102	12.0
	120	2,268	25.0
ts 11	0[d]		
	10	3,306	36.0
	30	2,703	29.0
	60	2,542	28.0
	120	1,805	20.0

[a] Specific infectivity of WT virus was 50,000 PFU/cpm, and that of *ts* 11 virus was 34,000 PFU/cpm.

[b] Trichloroacetic-acid insoluble counts per minute.

[c] Input, 8,900 cpm.

[d] Input, 9,189 cpm.

TABLE 5. *Immune reactivity of PEC from adolescent CD-1 mice inoculated with WT or ts 5 viruses as assessed by the MIF assay*[a]

Virus[b]	Antigens tested in MIF assay[c]	Distance of migration[d]	Mean migration index[e]
WT	Heart extract (280 μg), WT virus-inoculated mice	6.4	8 ± 1
	Heart extract (140 μg), WT virus-inoculated mice	32.2	42 ± 2
	Heart extract (280 μg), normal mice	69.4	90 ± 1
	WT virus lysate (10^8)	5.9	8 ± 1
	WT virus lysate (10^7)	40.9	53 ± 1
	HeLa cell lysate	72.8	94 ± 2
ts 5	Heart extract (280 μg), WT virus-inoculated mice	62.2	101 ± 2
	Heart extract (140 μg), WT-virus inoculated mice	65.0	103 ± 2
	Heart extract (280 μg), normal mice	66.2	104 ± 2
	WT virus lysate (10^8)	58.8	96 ± 1
	WT virus lysate (10^7)	59.7	98 ± 1
	HeLa cell lysate	58.1	95 ± 2

[a] Virus inoculation and PEC harvest methods and the assay are described in the text.

[b] Virus with which the mice that were the source of PEC were inoculated.

[c] Antigens tested per 0.1 ml were KCl extracts of heart tissues from CD-1 mice inoculated i.p. with 10^8 PFU of each virus per ml or heart tissues from normal mice and HeLa cell lysates from WT-inoculated or uninoculated cell cultures, both lysates diluted in RPMI 1640 to contain 160 μg/0.1 ml.

[d] Measurement of distance of migration was aided by an ocular grid in an inverted light microscope and expressed as the number of lines traversed by migrating cells. Each value represents the mean of 32 measurements taken from four equidistant points on 8 droplets.

[e] Mean migration index = (distance of cell migration with challenge antigen/distance of cell migration without challenge antigen) × 100 ± standard deviation, error calculated by the method of Paque et al. (18).

TABLE 6. *Capacity of WT and ts 5 viruses for induction of murine cardiac antigens in vivo which upon KCl extraction inhibit migration of immune (WT) PEC in an in vitro MIF assay*[a]

Expt no.[b]	Virus[c]	Mean migration index ± SD[d] at concn (μg of protein/0.1 ml of medium):			
		50	100	200	400
1	WT	84 ± 7	71 ± 4	61 ± 3	46 ± 3
	ts 5	96 ± 5	98 ± 5	100 ± 4	100 ± 4
2[e]	WT	102 ± 3	90 ± 5	31 ± 5	20 ± 5
	ts 5	101 ± 3	99 ± 5	96 ± 5	ND[f]

[a] See text for details of KCl extraction of murine cardiac tissue following i.p. inoculation of mice 8 days previously with 10^7 PFU of one virus and testing of these antigens in the in vitro agarose droplet MIF assay.

[b] KCl cardiac antigen extracts in experiments 1 and 2 represent six different preparations.

[c] Virus with which mice were inoculated before KCl-extracted murine cardiac antigens were obtained.

[d] Calculation of these indexes is given in the text, as is the statistical basis for accepting mean migration indexes of 65% or below as indicating positive inhibition. Antigen concentrations are given per 0.1 ml tested per well. SD, Standard deviation.

[e] The WT preparation was actually tested at 57, 115, 230, and 460 μg of protein per 0.1 ml instead of 50, 100, 200, and 400 μg of protein per 0.1 ml, respectively.

[f] ND = not done.

TABLE 7. *Serum interferon titers in mice after i.p. inoculation with WT or prototype ts mutants of coxsackievirus B3*

Time p.i.[a] (h)	Interferon titer after inoculation with:[b]			
	WT	*ts* 1	*ts* 5	*ts* 11
24	<10	<10	<10	<10
48	40	<10	<10	<10

[a] Inoculation dose was 10^7 PFU per mouse i.p.

[b] One unit of interferon is that amount which reduces vesicular stomatitis virus plaque number by 50%. The titer is the reciprocal of the highest dilution at end point.

ts 11 viruses on days 0 and 7. On day 14, 10 mice of each group were sacrificed, and their hearts were examined for myocardial lesions. All remaining mice in each group were inoculated i.p. with 10^7 PFU of WT virus. On day 21, all mice were sacrificed and their hearts were removed for histopathological examination. Mice receiving WT virus only contained cardiac tissue which exhibited myocardial lesions. All mice which received two injections of one of the three prototype *ts* mutants before inoculation with WT virus had no evidence of myocarditis. Thus, these three *ts* mutants served as viable vaccine strains in protection against myocarditis.

Susceptibility of newborn mice to WT, *ts* mutants, and revertants of *ts* mutants. Pathogenicity of *ts* mutants thus far has been measured by capacity for induction of myocarditis in adolescent CD-1 mice. Newborn CD-1 mice were inoculated by several routes with WT, *ts* mutants, and revertants. Neonates were unable to survive after inoculation with WT, *ts* 1R, *ts* 5R and *ts* 11R viruses, whereas neonates challenged with *ts* 1, *ts* 5, and *ts* 11 exhibited variable degrees of survival (Table 8). Thus, age of the host is critical in assessing pathogenicity of a virus.

DISCUSSION

Myocarditis has been induced in the murine model by various routes of inoculation with coxsackieviruses B1-B5 (3, 8, 14). The histopathology of cardiac tissue taken from these mice is similar to that observed in human cardiac tissue taken from individuals who had myocarditis associated with coxsackievirus B3 (3). Although immunological responses most certainly play a role in the disease process after infection with these viruses (see below), there is obviously a genetic basis for the cardiotropism expressed by coxsackievirus B1-B5. For example, coxsackievirus B6 has not been associated with myocarditis in either animals or humans, and yet this virus shares a common group antigen with all other group B coxsackieviruses (19). Furthermore, a variant of coxsackievirus B3, strain Nancy, produces little or no myocarditis after inoculation into adolescent CD-1 mice (C. J. Gauntt, M. D. Trousdale, D. R. LaBadie, and R. E. Paque, Abstr. Annu. Meet. Am. Soc. Microbiol., S282, p. 326). Our approach toward understanding the genetic basis for induction of myocarditis was the isolation and characterization of *ts* mutants from a myocarditic coxsackievirus B3 strain Nancy parent. None of 10 *ts* mutants of the latter virus induced myocarditis in adolescent CD-1 mice. These *ts* mutants separated into three groups on the basis of complementation analyses at 39.5°C (24); thus, variants with *ts* defects in at least three separate genes are deficient in this biological property of the parent virus. Revertants from 9 of the 10 *ts* mutants were isolated, and only 5 induced myocarditis in mice. These data show that reversion of a *ts* mutant to replication at 39.5°C is not covariant with reacquisition of pathogenicity (myocarditis). The ease with which these revertant variants were obtained suggests complete restoration of the replication defect, but only partial restoration of the defect in pathogenicity in the genes defined for group II and III virus variants.

TABLE 8. *Lethality of ts mutants and ts mutant revertants of coxsackievirus B3 for newborn mice*[a]

Virus variant	No. dead[b]/no. inoculated	% Survival
None[c]	5/107	95
WT	83/83	0
ts 1	20/59	66
ts 5	29/31	6
ts 11	32/53	40
ts 1 revertant	46/46	0
ts 5 revertant	48/48	0
ts 11 revertant	72/72	0

[a] Newborn mice (<24 h of age) were inoculated by multiple routes (i.p., subcutaneously, and intracerebrally) with 10^6 PFU as listed in the text.

[b] Death occurred between 1 and 27 days p.i.; surviving mice were sacrificed at 30 days p.i.

[c] Inoculated with minimum essential medium as trauma controls.

Some strains of poliovirus are reported to lack neurovirulence for primates because of their inability to adsorb to receptors on primate nervous tissue (15). The amyocarditic property exhibited by the *ts* mutants was not the result of an incapacity of the *ts* 11 mutant to attach to adolescent mouse heart tissue, because both WT and *ts* 11 mutant viruses adsorbed equally well to adolescent heart tissue.

We examined several factors which may contribute to the reduced pathogenicity of *ts* mutant viruses, including reduced capacity to replicate at 37°C in CD-1 mice and heat stability of the capsids of particles of a given virus variant. Compared with WT virus, the three *ts* mutants all have reduced capacity for replication at 37°C, reduced replicative capacity in heart tissues in vivo, and reduced heat stability of the capsid. Lack of replication may explain their inability to induce interferon. The *ts* 5R revertant exhibits reduced replicative capacity in heart tissue and reduced capsid stability, yet *ts* 1R and *ts* 11R share these latter two properties and can induce myocarditis. Thus, there are, apparently, other unknown properties required of a virus variant for pathogenicity, and these are currently under investigation.

As mentioned earlier, host immune responses have been cited as important in virus-induced myocarditis (3, 26, 27). Rager-Zisman and Allison have reported that both circulating antibody and CMI responses are important in recovery from coxsackievirus B3 infections (20). They demonstrated protection of suckling mice against coxsackievirus B3 inoculation by passive immunization with specific antibody and immune PEC elicited by the same virus. We demonstrated that each of the prototype *ts* mutant viruses can serve as experimental vaccine strains in adolescent CD-1 mice and provide protection against a challenge of WT virus which is suffi-

cient to induce myocarditis.

CMI responses in mice were assessed by the MIF assay. Inhibition of macrophage migration in semisolid agarose droplets by MIF has been demonstrated to correlate well with CMI (9). CMI in mice inoculated with WT or *ts* 5 viruses were quite different as assessed by the MIF assay. MPEC from mice inoculated with WT virus reacted in an immunospecific manner with KCl-extracted antigens from cardiac tissue of mice inoculated with WT virus, in agreement with previously reported results (18). Also in agreement with the previous data on purified virions (18) was the finding that the latter MPEC reacted with antigens in WT virus HeLa cell lysates. *ts* 5 virus-inoculated mice yielded MPEC which failed to be inhibited by either set of antigens from WT virus-infected, KCl-extracted cardiac tissues or crude HeLa cell lysates. Thus, by one assay method, *ts* 5 virus failed to induce a demonstrable CMI response as assessed by an in vitro assay. Control antigens from KCl extracts of normal heart tissue and uninoculated HeLa cells proved to be unreactive against MPEC from mice inoculated with either WT or *ts* 5 viruses, demonstrating the specificity of the in vitro immunobiological test. Woodruff and Woodruff (27) reported that the severity of myocarditis is reduced in coxsackievirus-infected mice which have a T-cell deficiency. Recently, Wong et al. (26) demonstrated that T lymphocytes in coxsackievirus B3-inoculated mice were cytotoxic against both uninfected and coxsackievirus B3-infected syngeneic fibroblasts. Cytotoxic activity against virus-infected syngeneic fibroblasts was also demonstrated with nonimmune T cells; however, lysis occurred at a significantly reduced level (26). Their data also suggested that virus infection stimulates a CMI response directed at antigens produced in the heart. The amyocarditic *ts* 5 mutant failed to induce antigens in cardiac tissue of mice at 8 days p.i. which would react immunospecifically with sensitized MPEC from WT-inoculated mice. We have found that an amyocarditic variant of coxsackievirus B3 also does not induce production of cardiac antigens which inhibit migration of MPEC from WT virus-immunized mice in the in vitro agarose droplet MIF test (C. J. Gauntt, M. D. Trousdale, D. R. L. LaBadie, R. E. Paque, and T. Nealon, J. Med. Virol., in press). However, pertinent to this discussion is a recent finding that *ts* 5R, which is amyocarditic, induces antigens in cardiac tissue which will induce a specific immune reaction in MPEC taken from WT virus-inoculated mice (Gauntt, Trousdale, Nealon, and Paque, unpublished data). Thus, the two properties of these viruses, namely capacity for induction of myocarditis and capacity for induction of KCl-extractable cardiac antigens which react with MPEC from WT virus-inoculated mice in the MIF assay, are not covariant. Other investigators have found that mice immunosuppressed with cyclophosphamide develop severe myocarditis upon inoculation with coxsackievirus B3 (20; Paque and Nealon, unpublished data). Because cyclophosphamide suppresses both the humoral and CMI responses (16), these present findings suggest that coxsackievirus B3-induced myocarditis may not result entirely from an immunological mechanism.

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