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Characterization and Myocarditic Capabilities of Coxsackievirus B3 Variants in Selected Mouse Strains

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Two variants of coxsackievirus B3 (CVB3) were compared with the original myocarditic parent variant ($CVB3_m$) for myocarditic properties in several strains of mice. The *ts*1R variant produced little to no myocarditis in any of the nine mouse strains examined. The *ts*10R variant and $CVB3_m$ could be differentiated on the basis of the extent of myocarditis induced in mice of selected *H-2*^{b} and *H-2*^{k} haplotypes and in the female versus the male responses of two other inbred strains. Virus quantities recovered from the hearts of myocarditic mice did not correlate with the extent of disease. The three variants could not be differentiated on the basis of: (i) rate and extent of adsorption to heart tissue homogenates, (ii) kinetic neutralization rates with antiserum directed against $CVB3_m$, (iii) ^{125}I labeling of surface regions of polypeptides on purified particles, or (iv) rates of heat inactivation of infectivity at 50°C. These data suggest that differences in pathogenicity cannot be attributed to major alterations in capsid polypeptides. Oligonucleotide fingerprint maps of T_1 RNase digests of the genomes of purified particles of the three CVB3 variants showed distinct differences. Thus, the extent of myocarditis induced by CVB3 variants in a mouse model is affected by some subtle expression of the genome, presumably not involving capsid polypeptides, as well as by the haplotype and sex of a given mouse host species.

Several mouse models, with both semi-inbred and inbred strains of mice, have been established to study the mechanism(s) of induction of myocarditis by coxsackieviruses B1 through B5 (6, 8, 11, 14, 16, 23, 26, 31, 33–35). Intraperitoneal inoculation of adolescent or adult mice with coxsackievirus B3 (CVB3) results in the infection of several organs with the production of maximum myocarditis between days 6 and 9 postinoculation (p.i.). The time required for development of myocarditis, the extent of myocarditis induced, and the amounts and time course of virus isolation from heart tissues vary (14, 27, 28, 35). These studies were carried out in different laboratories; therefore, it is not possible to ascertain whether variations in the results reflect genetic differences in mouse strains, genotypes of the CVB3 variants used, or both (1, 28).

In a previous study (14), we showed that a myocarditic variant of CVB3 ($CVB3_m$) could be differentiated from an amyocarditic variant on the basis of antigens induced in heart tissues to which CVB3-sensitized lymphoid cells reacted in vitro. In another study (39), we demonstrated that both myocarditic and amyocarditic revertant variants could be selected from amyocarditic temperature-sensitive (*ts*) mutants which originated from the $CVB3_m$ parent. In the present report, we extended the latter studies to assess the comparative myocarditic capabilities of two *ts* revertant variants and the original $CVB3_m$ parent in several strains of mice. The amyocarditic behavior of one *ts* revertant (*ts*1R) of CVB3 was expressed in all mouse strains examined. The results also show that the genetic background of the host can be a factor in determining the extent of myocarditis induced by a particular CVB3 variant. By using hosts with different *H-2* and *Lyt* haplotypes, it was possible to distinguish between two myocarditic variants of CVB3. Although some of our data agree with previous reports indicating that males of many mouse strains are more susceptible to the induction of myocarditis than are females (4, 8, 12), we present data to show that females of some mouse strains can be more susceptible than males to a certain CVB3 variant.

MATERIALS AND METHODS

Cell culture and viruses. HeLa cells used in these studies were cultured and used in plaque assays as previously described (38). The origin, propagation, and plaque assay of the parent $CVB3_m$ have also been described (38). The isolation of revertants (variants with efficiency of plating at 39.5/efficiency of plating at 34°C ≥ 1.0) from *ts* mutant variants of $CVB3_m$ was previously described (39). The revertants, *ts*1R and *ts*10R, were isolated from *ts*1 and *ts*10 mutant viruses, respectively, and plaque purified three times before the preparation of stock viruses. Virus stocks of 5×10^8 to 2×10^9 PFU per ml were prepared in HeLa cells incubated in virus growth medium (minimal essential medium [MEM] containing 1% heat-inactivated fetal bovine serum and gentamicin at 50 μg/ml) at 34°C (*ts*10 virus) or 37°C (*ts*1R, *ts*10R, and $CVB3_m$ viruses).

Mice. CD-1 semi-inbred mice were purchased from Charles River Breeding Laboratories, Inc., Boston, Mass., as breeding pairs. Of the 14 inbred strains of mice used, 8 were maintained in our departmental animal facility, and the remainder were purchased from Jackson Laboratories, Bar Harbor, Maine. Congenic and congenic-resistant mice with C57BL/6 backgrounds were kindly provided by E. A. Boyce, Sloan Kettering Memorial Cancer Institute, New York, N.Y. Adolescent mice (4 to 6 weeks of age) of both sexes were used in experiments except where a single gender is designated. Mice were inoculated intraperitoneally with 10^7 or 10^8 PFU in 0.2 ml of a given virus and sacrificed by cervical dislocation at day 8 p.i.

Processing of heart tissues for assay of virus contents and

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histology. Hearts from mice were immediately removed after death, and the apical one-third of the heart from each animal in a given group was taken. These tips were pooled, weighed, Dounce homogenized, and frozen and thawed three times, and a 10% suspension was prepared in MEM for plaque assay. The remaining two-thirds of each heart was fixed in 10% Formalin, embedded in paraffin, and stained with hematoxylin-eosin (39).

Assessment of myocarditis. At least two or more adjacent sections of each heart were examined microscopically for the presence of myocardial lesions, which consisted of focal, irregularly shaped areas containing necrotic cells and interstitial spaces infiltrated with numerous mononuclear and polymorphonuclear leukocytes (14, 39, 45).

Preparation and purification of ^{32}P-labeled virus particles, extraction of RNA and oligonucleotide fingerprint mapping techniques. Approximately 2.5×10^8 HeLa cells in monolayer cultures (38) were challenged with 50 to 100 PFU of each virus per cell. After 1 h for adsorption of virus at 34°C, the inoculum was removed, and phosphate-free MEM supplemented with complete MEM to 1/10 of the final volume and 1% heat-inactivated fetal bovine serum was added. These cultures were returned to a humidified CO_2 incubator at 34°C. At 2 h p.i., orthophosphate-free ^{32}P was added at 50 μCi/ml. The cells were harvested at 12 h p.i. and processed to obtained virus particles, which were purified as previously described (15). Virus particles were treated with proteinase K (Beckman Instruments, Inc., Palo Alto, Calif.) and solubilized with sodium dodecyl sulfate, and the RNA was extracted with a 25:24:1:0.01 (wt/wt) mixture of phenol–chloroform–isoamylalcohol–8-hydroxyquinoline. After two precipitations in 2 volumes of ethanol at −20°C for 18 h, the RNA was suspended in TE buffer (0.02 M Tris-hydrochloride [pH 7.4] and 0.002 M EDTA). Virus RNA was digested with 10 U of T_1 RNase and the resulting oligonucleotides were separated by two-dimensional electrophoresis by the methods of Trent et al. (37).

Purification, surface-iodination, and polyacrylamide gel electrophoresis of virus particle polypeptides. Preparations of *ts*10R, *ts*1R, and $CVB3_m$ virus particles were obtained as previously described (15). After banding in CsCl, the virus preparations were extensively dialyzed against phosphate-buffered saline (PBS), and surface regions of the virus particle polypeptides were iodinated (with ^{125}I) by a lactoperoxidase procedure (13). These preparations were desalted with a Bio-Gel P-6DG gel column (0.6 by 25 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted in the following buffer: 0.05 M Tris (pH 7.0), 0.145 M NaCl, and 0.004 M EDTA. The initial 2 to 3 fractions in the void volume which contained ^{125}I-labeled virus were pooled and layered over a 4-ml cushion of 20% sucrose in PBS containing 0.018 M KI. The virus particles were pelleted by centrifugation at 189,000 × *g* for 90 min in a Beckman SW50.1 rotor. The virus pellet was suspended in 0.2 ml of dissolving buffer (0.0625 M Tris [pH 6.8], 2% sodium dodecyl sulfate, 2% beta-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue). After being boiled for 4 min, 20 μl containing ca. 10,000 cpm of each virus variant was applied to a discontinuous sodium dodecyl sulfate-polyacrylamide slab gel system, essentially as described by Laemmli (25), except that the separating gel contained 12.5% acrylamide. The samples were electrophoresed at 10 mA of constant current (~3 to 3.5 h) until the dye front entered the separating gel, and then a constant current of 30 mA was applied until the dye front arrived at the edge of the 10-cm separating gel (~3 h). The gel was fixed in a mixture of methanol-water-glacial acetic acid (5:4:1) for 1 h, rinsed in a water–1% glycerol mixture for 2 h, and shrunk ca. 12% in a methanol–1% glycerol mixture. The gel was dried by vacuum and exposed to Kodak XAR film backed by a DuPont Cronex Lightning-Plus B1 intensifying screen.

Kinetic neutralization test. The kinetic neutralization test was performed as outlined by Habel (17), with antiserum obtained at 11 days p.i. from two adult female baboons (*Papio papio*) after a single inoculation with $CVB3_m$ (32). Serum from the two baboons was pooled, heat inactivated at 56°C for 30 min, and diluted 1:1,280 for use in these experiments.

Attachment of CVB3 variants to disrupted heart tissues. Purified particles of *ts*10, *ts*1R, *ts*10R, and $CVB3_m$ containing [^{35}S]methionine were prepared as described above except that at 3 h p.i., the virus growth medium was removed, and the cells were washed once with MEM containing 1/20 the normal amount of amino acids. [^{35}S]methionine (>600 Ci/mmol; Amersham, Corp., Arlington Heights, Ill.) was added to 5 μCi/ml to the virus growth medium, and incubation was continued at 34°C for an additional 6 h. Virus particles were purified as previously described, except that they were twice banded in CsCl gradients, and the final band was extensively dialyzed against PBS. Specific infectivities were similar for the four preparations and varied from 32 to 41 PFU/cpm. The hearts from four 6-week-old CD-1 males were rinsed, the auricles and pericardia were removed, and the remaining tissues were extensively minced with scissors and Dounce homogenized with six strokes of a tight-fitting Dounce homogenizer. The disrupted tissue was washed thrice in cold PBS and centrifuged at 1,200 × *g* at 4°C. Fifty percent of the cells in the resulting suspension were viable as determined by their exclusion of trypan blue. Cells and virus (PFU) were mixed in approximately a 1:1 ratio in a final volume of 0.2 ml in duplicate samples. The samples were placed at 37°C in polyethylene tubes, harvested by vacuum filtration onto Whatman 3MM filters at 0, 5, 10, 20, and 40 min postmixing, washed extensively with PBS, and dried. The counts per minute were determined in a Mark III beta spectrometer. All counts were corrected for the low background of nonspecific attachment of labeled viruses to filters.

RESULTS

Comparative assessment of the myocarditic potentials of *ts*1R, *ts*10R, and $CVB3_m$ in mouse strains. The induction of myocarditis presumably depends upon the genotype of the host and the virus variant (44). Experiments were done to determine whether the severity of myocarditis induced by a particular isogenic CVB3 variant(s) correlated with *H-2* haplotype, LYT phenotype, or gender of a particular strain of mouse. The inoculation of *ts*1R into several strains of mice did not reproducibly induce significant levels of myocarditis in any of the mouse strains tested, whether semiinbred or inbred carrying *H-2* haplotypes *b*, *k*, or *d* (Table 1). Gender of the mouse was not a factor in myocarditis induction by this virus variant. The results with the myocarditic *ts*10R and parent $CVB3_m$ variants provided different results (Table 2). Groups of mice were either of both sexes or male only. The data show that titers of virus present in the heart tissues of mice inoculated with *ts*10R or $CVB3_m$ did not correlate with the severity of myocarditis induced. Distinct differences in capabilities of $CVB3_m$ and *ts*10R for the induction of myocarditis were found in several different strains of mice. $CVB3_m$ was significantly more myocarditic than was *ts*10R in 129/J mice, whereas the *ts*10R variant was

TABLE 1. Myocarditic potential of *ts*1R in different mouse strains[a]

Mouse strain	*H-2* haplotype	LYT phenotype	Virus titer in heart tissue (PFU/g × 10^{-2})[b]	Total avg. no. of lesions per section ± SEM[c]
CD-1	Unknown	Unknown	<0.4, <0.4, <0.5 <0.6, 117, ND	0.1 ± 0.01
C57BL/6J	*b*	1,2,2.2,3.2	<0.8(♀), 17.1(♂) 1,260	0.3 ± 0.08
C57BL/6 LYT 1.1	*b*	1.1,2.2,3.2	<0.7, <4.4 ND	0.1 ± 0.04
C57BL/6 LYT 2.1	*b*	1.2,2.2,3.2	<0.9 19.3	0
C57BL/6 LYT 2.1 LYT 3.1	*b*	1.2,2.1,3.1	13.0 ND	0.9 ± 0.5
BALB/c	*d*	1.2,2.2,3.2	<0.6(♀), <0.5(♂), 2.6 190, ND	0
C57BL/6 *H-2*^{k}	*k*	1.2,2.2,3.2	73.1(♀, 796(♂), 227 ND	0.5 ± 0.18
C57BL/6 *H-2*^{k} CE LYT 2.1:DS	*k*	1.2,2.1,3.2	60.9, ND	0.2 ± 0.05
C3H-CE LYT 1.2:DS	*k*	1.2,2.2,3.2	197, ND	0.5 ± 0.14

[a] Groups of 4 to 7 mice per experiment were inoculated intraperitoneally with 10^7 or 10^8 PFU per mouse and sacrificed at day 8 p.i. Virus contents were assessed in pooled heart tips. Unless noted, groups contained animals of both genders.
[b] Mean virus titers in separate experiments. ND, Not determined.
[c] Total average lesion score for all animals in all experiments.

significantly more myocarditic than was $CVB3_m$ in mice of the C57BL/6 *H-2*^{k} and C57BL/6 *H-2*^{k} CE LYT 2.1:DS strains. From this cursory survey of mouse strains, it appeared that $CVB3_m$ expressed myocarditic potential to a greater extent in *H-2*^{b} haplotypes, whereas *ts*10R expressed greater myocarditic potential in *H-2*^{k} haplotypes. The *Lyt* haplotype did not appear to influence the severity of myocarditis induced by either *ts*10R or $CVB3_m$. Within each haplotype, strains carrying the same LYT phenotype varied greatly in their myocarditic response to either virus variant. Thus, an *Lyt* locus was not found to be associated with susceptibility or resistance to virus induction of myocarditis. These results indicated that mice responded differently to $CVB3_m$ and *ts*10R variants, and although factors associated with *H-2* and *Lyt-1*, *-2*, and *-3* loci made important contributions to the disease, undetermined factors which did not appear to be related to these loci were important in the pathogenesis of CVB3-induced myocarditis.

Effect of gender on the extent of myocarditis induced by $CVB3_m$ or *ts*10R in different strains of mice. Variability in the extent of myocarditis induced in mice by CVB3 variants has been reported to be due in part to gender of the animal, with males reportedly being more susceptible (19, 21, 43, 44). Our data showed that this generalization is not valid; females of some strains were more susceptible than males to the induction of myocarditis by CVB3 variants. The gender most susceptible to the induction of myocarditis by $CVB3_m$ or *ts*10R differed among several mouse strains (Table 3). The parent virus $CVB3_m$ induced the most severe myocarditis in females of the SWR/J and B10-D2/$^{NS}_{NU}$ strains, whereas *ts*10R was most myocarditic in females of the SWR/J strain. From data on both gender and mouse strain differences, it was clear that susceptibility to the induction of myocarditis by $CVB3_m$ could be differentiated from that induced by *ts*10R by severity: (i) in 129/J mice, only males were susceptible to $CVB3_m$, whereas either gender of this strain was relatively insusceptible to *ts*10R; (ii) in B10-D2/$^{NS}_{NU}$, mice, males and females were both highly susceptible to $CVB3_m$, whereas only males were highly susceptible to *ts*10R; (iii) in BALB/c mice, either gender was relatively insusceptible to $CVB3_m$, whereas males were more susceptible than females to *ts*10R-induced myocarditis; and (iv) in C57BL/6 *H-2*^{k} mice, $CVB3_m$ induced minimal myocarditis in mice of either gender, whereas *ts*10R induced significantly more myocarditis in male mice. In summary, the 129/J and B10-D2/$^{NS}_{NU}$ strains were useful in clearly distinguishing between $CVB3_m$ and the *ts*10R variant. Although virus titers in heart tissues did not correlate with the extent of myocarditis in most mouse strains, we noted that in both strains in which females were more susceptible than males to either $CVB3_m$ or *ts*10R, virus titers were higher in heart tissues from the female mice.

Properties of the CVB3 variants. We next compared several properties of these CVB3 variant viruses to determine whether one or more properties might account for the reduced myocarditic capabilities of the *ts*1R variant.

(i) Antigenic alterations in capsid surface structures of the variants. Although the mechanism of neutralization of picornavirus is unknown, it has been shown for coxsackievirus B3 that substructures containing polypeptide VP2 induce neutralizing antibodies (2, 22). It was of interest to ascertain whether antigenicity in capsid structures of *ts*1R, *ts*10R, and $CVB3_m$ virions differed, since large changes in surface

TABLE 2. Comparison of mycocarditic potentials of $CVB3_m$ and *ts*10R variants in different strains of mice[a]

Mouse strain[b]	*H-2* haplotype	LYT phenotype	Virus contents and extent of myocarditis in mice inoculated with:			
			$CVB3_m$		*ts*10R	
			Virus titer in heart tissue (PFU/g × 10^{-2})	Avg. no. of lesions per section ± SEM	Virus titer in heart tissue (PFU/g × 10^{-2})	Avg. no. of lesions per section ± SEM
CD-1	Unknown	Unknown	109.3	0.8 ± 0.2	76	1.0 ± 0.2
SWR/J (♂)	*q*	1.2,2.2,3.2	1,140	22.3 ± 6.6	130	10.6 ± 3.3
129/J (♂)	*b*	1.2,2.2,3.2	2,900	29.1 ± 10.6[d]	2.3	0.5 ± 0.2[d]
C57BL/6J	*b*	1.2,2.2,3.2	97,142	7.8 ± 2.5	3,921	4.7 ± 1.8
C57BL/6 LYT 1.1	*b*	1.1,2.2,3.2	1,563	6.6 ± 2.3	3,370	1.1 ± 0.5
C57BL/6 LYT 2.1	*b*	1.2,2.1,3.2	399	2.3 ± 0.8	196	1.6 ± 0.5
C57BL/6 LYT 2.1, LYT 3.1	*b*	1.2,2.1,3.1	470	4.0 ± 0.5	1,131	0.8 ± 0.3
BALB/c	*d*	1.2,2.2,3.2	1,563	1.8 ± 0.7	521	2.5 ± 0.5
DBA/2J (♂)	*d*	1.1,2.1,3.2	8.6	2.7 ± 0.7	16	2.0 ± 0.9
BIO-D2/$_{NU}^{NS}$ (♂)	*d*	1.2,2.2,3.2	650	36.0 ± 16.1	420	37.4 ± 12.2
C57BL/6*H-2*^{k}	*k*	1.2,2.2,3.2	1,078	1.4 ± 0.2[e]	5,204	7.5 ± 1.4[e]
CBA/T6J (♂)	*k*	1.1,2.1,3.2	303	1.7 ± 0.9	28	0
C58/J (♂)	*k*	1.2,2.1,3.1	ND[c]	12.7 ± 4.4	ND	31.0 ± 11.2
C57BL/6 *H-2*^{k} CE LYT 2.1:DS	*k*	1.2,2.1,3.2	1,020	2.2 ± 0.2[e]	681	6.2 ± 0.7[e]
C3H CE LYT 1.2:DS	*k*	1.2,2.1,3.1	10,300	3.9 ± 0.9	6,410	6.1 ± 0.8

[a] Mice were inoculated with 10^6 (♂), 10^7, or 10^8 PFU per mouse and sacrificed at day 8 p.i. A minimum of five mice per group and up to three separate experiments per mouse strain are reported.
[b] Both sexes were used unless noted; males only, ♂.
[c] ND, Not determined.
[d] Student's *t* test differences: $P < 0.01$.
[e] Student's *t* test differences: $P < 0.05$.

structures could affect the attachment and perhaps the penetration of virus particles into cells. Kinetic neutralization tests with hyperimmune antiserum prepared against $CVB3_m$ were used. The results (Fig. 1) show that the kinetics of virus neutralization were nearly identical for all viruses, including the immediate parent of *ts*10R, i.e., *ts*10. Thus, antigenic alterations in capsid surface structures of these four viruses were not detected by direct neutralization of infectivity.

(ii) Adsorption of CVB3 variants to a heart tissue homogenate. Experiments were next performed to directly measure the attachment of purified virus particles of *ts*1R, *ts*10R, and $CVB3_m$ to receptors on homogenized heart tissues and cell membranes. Although VP2 was apparently antigenically similar among $CVB3_m$ and the two revertant variants, it is possible that structural changes in the polypeptide(s) near the determinants involved in evoking neutralizing antibodies could affect the rate and extent of attachment of particles to their specific cell receptors. Purified [^{35}S]methionine-labeled virus particles of *ts*1R, *ts*10R, and $CVB3_m$ were mixed with samples of homogenized adolescent CD-1 mouse heart tissue. All virus variants attached at similar relative rates of adsorption (data not shown). Attachment was virtually complete during the first 5 min of the 40-min period, and elution did not occur during the subsequent 35 min. The tissue homogenates adsorbed between 9.1 and 11.4% of each virus challenge inoculum, as assessed by counts per minute in bound ^{35}S. Thus, the amyocarditic nature of *ts*1R was apparently not due to a reduced rate of virus attachment to receptors on heart cells.

(iii) Efficiency of plating. The reduced capability of *ts*1R for the induction of myocarditis could possibly be due to reduced replication of the virus at temperatures approximate to that of the mouse heart. Titers of *ts*1R virus from heart tissues of *ts*1R-inoculated mice appeared to be lower than titers of either *ts*10R or $CVB3_m$ in heart tissues (Table 1). To assess the possibility of reduced virus growth, the efficiency of plating of *ts*1R, *ts*10R, and $CVB3_m$ viruses on HeLa cells at 39.5 and 34°C was determined. The efficiency of plating of *ts*1R, *ts*10R, and $CVB3_m$ were respectively, 0.71, 0.89, and 0.64 in one experiment and 1.21, 1.30, and 1.36 in a second experiment. Plaque sizes of all viruses were similar at the two temperatures. Thus, the capacity for replication at 39.5°C did not appear to be significantly different among these viruses; therefore, we cannot acount for the amyocarditic nature of *ts*1R by a reduced capacity for replication at a high temperature such as might be encountered in heart tissues in vivo.

(iv) Heat inactivation. The stability of infectivity in *ts*1R, *ts*10R, and $CVB3_m$ virions in MEM to incubation at 50°C was measured by plaque assay. Curves constructed on the basis of percentage of surviving virus after 1, 3, 5, 7, or 9 min of incubation showed no appreciable differences among rates of inactivation for these three viruses (data not shown). Only 2 to 4% of infectivity remained after 9 min of incubation. Alterations in capsid polypeptides which might lead to

TABLE 3. Importance of gender on the induction of myocarditis by $CVB3_m$ or *ts*10R variants in different strains of mice[a]

Mouse strain	Sex	Virus contents and extent of myocarditis in mice inoculated with:			
		$CVB3_m$		*ts*10R	
		Virus titers in heart tissue (PFU/g × 10^{-3})	Avg. no. of lesions per section ± SEM[b]	Virus titers in heart tissue (PFU/g × 10^{-3})	Avg. no. of lesions per section ± SEM
CD-1	♀	1.2	1.0 ± 0.6	<0.1	2.2 ± 1.3
	♂	4.7	0.5 ± 0.2	2.4	1.1 ± 0.3
SWR/J	♀	708,000	76.8 ± 14.7*	740	111.8 ± 23.1**
	♂	1,140	22.3 ± 6.6*	130	10.6 ± 3.3**
129/J	♀	19	1.1 ± 0.6*	30	0.1 ± 0.1
	♂	2,900	29.1 ± 10.6*	2.8	0.5 ± 0.2
CBA/T6J	♀	1,450	35.8 ± 15.9†	290	13.4 ± 5.3††
	♂	303	1.7 ± 0.9†	28	0 ± 0††
BIO-D2/$_{NU}^{NS}$	♀	6,300	78.9 ± 19.8	<10	1.1 ± 0.5*
	♂	650	36.0 ± 16.1	420	37.4 ± 12.2*
C57BL/6J	♀	3.2	2.6 ± 1.0†	0.2	0.3 ± 0.7†
	♂	194	17.2 ± 6.3†	7.8	12.7 ± 5.1†
BALB/c	♀	0.3	1.3 ± 0.4	0.2	0.5 ± 0.3†
	♂	0.6	0.6 ± 0.3	0.3	3.4 ± 1.3†
C57BL/6	♀	2.1	0.5 ± 0.2	9.5	3.1 ± 1.2†
H-2^k	♂	0.6	1.5 ± 0.3	2.3	13.3 ± 3.2†

[a] Mice were inoculated with 10^6 PFU (first five groups listed) or 10^7 to 10^8 PFU (last three groups) and sacrificed at day 8 p.i.

[b] Average number of lesions per section, with two sections per heart and a minimum of five mice per group. Student's two-tailed *t* test, for male versus female of a particular strain, average lesion number per section significantly different at: *, $P < 0.01$; **, $P < 0.001$; †, $P < 0.05$; and ††, $P < 0.02$.

instability in the capsid structure and decreased virion stability were not detected among these viruses by this technique. In this case, the relative heat instability of *ts*1R compared with that of *ts*10R or $CVB3_m$ does not appear to be a factor in the amyocarditic nature of *ts*1R.

(v) Oligonucleotide fingerprint maps of $CVB3_m$, *ts*10, *ts*10R, and *ts*1R variants. To determine whether differences exist at the subgenomic level among these CVB3 variants, the genomic RNAs were analyzed by oligonucleotide fingerprinting. The results (Fig. 2 and 3) show that there are several oligonucleotide differences among the variants. The $CVB3_m$ parent genome differs from the *ts*10 genome by possessing one additional oligonucleotide, no. 5 (Fig. 3). The genome of *ts*10R, a virus isolated from a stock of *ts*10 virus, differed from the *ts*10 parent virus in three oligonucleotide changes: no. 4 disappeared, and no. 1 and 3 appeared, suggesting two RNA sequence changes recognized by T_1 RNase. The *ts*10R and *ts*1R variants differed from each other in seven oligonucleotides: *ts*10R exclusively possessed oligonucleotides 1 and 3, whereas *ts*1R exclusively possessed no. 2, 6, and 7, but shared no. 4 with all variant genomes except *ts*10R and shared no. 5 with $CVB3_m$. These limited analyses did not permit us to describe an oligonucleotide pattern for either a myocarditic or an amyocarditic virus variant.

(vi) Surface labeling profiles of polypeptides in purified virus particles $CVB3_m$, *ts*1R, and *ts*10R. Alterations in the gross molecular structure of virus particle polypeptides could lead to surface changes on particles. Lactoperoxidase-catalyzed, ^{125}I-labeled, purified virus particles were solubilized and electrophoresed on a 12.5% polyacrylamide slab gel (Fig. 4). No major qualitative changes were found in the

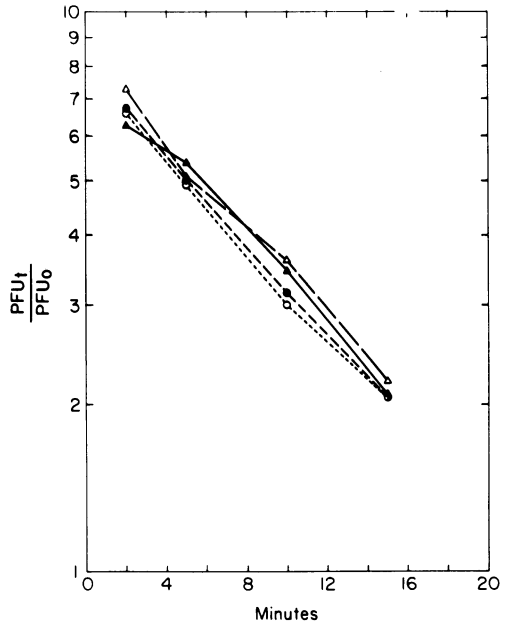

FIG. 1. Kinetic neutralization of *ts*1R, *ts*10R, *ts*10, and $CVB3_m$ with antiserum prepared against $CVB3_m$. Mixtures of virus and dilute antiserum were placed in a 37°C water bath. At the indicated intervals, samples were withdrawn from the virus-antibody mixture and diluted 1:1,000 in MEM. A 0.1-ml volume was inoculated onto cells, and the plaque assay was then performed. Neutralization curves for: *ts*10R virus (○– – –○), *ts*1R virus (●– –●), *ts*10 virus (△— —△), and $CVB3_m$ virus (▲——▲).

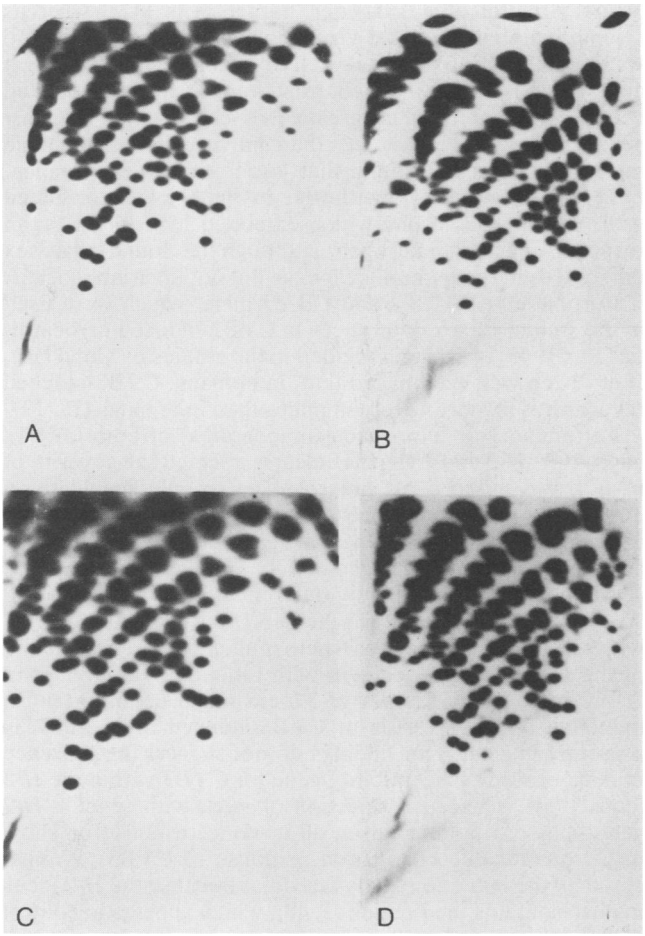

FIG. 2. Oligonucleotide fingerprint maps of RNase T_1 digests of ^{32}P-labeled genomic RNA extracted from purified $CVB3_m$, *ts*10, *ts*10R, or *ts*1R virus particles. Maps are shown for (A) $CVB3_m$, (B) *ts*10, (C) *ts*1R, and (D) *ts*10R. The polyadenosine tail appears as a streak in the lower left of each panel.

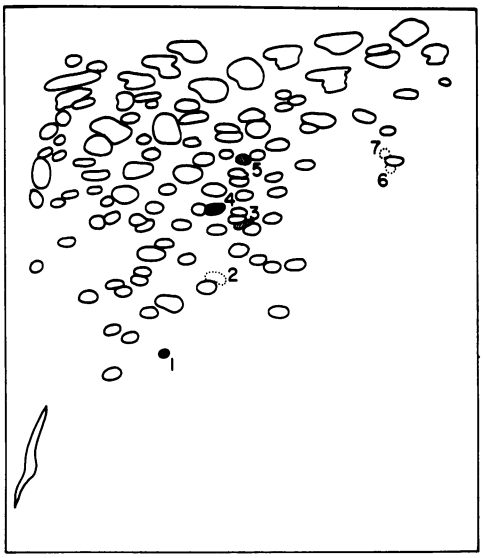

FIG. 3. Tracing of oligonucleotide fingerprint map of *wt* genome oligonucleotides. The numbered oligonucleotides indicate differences in the presence or absence of oligonucleotides in maps of the other three viruses, specifically: oligonucleotides 1 and 3 are present only in the *ts*10R map; oligonucleotides 2, 6, and 7 are present only in the *ts*1R map; oligonucleotide 4 is present in all maps except that of *ts*10R; and oligonucleotide 5 is present only in the maps of *ts*1R and $CVB3_m$.

migration mobilities among these three variants. Purified particles contained VP1, VP2a, and VP3, as previously demonstrated for $CVB3_m$ (15), and the extent of surface labeling was qualitatively similar for the three variants. A quantitative ^{125}I labeling difference which was somewhat variable among different virus preparations was observed for VP2b among these variants. The relative extent of surface labeling of VP2b was lower for *ts*10R virus particles than for *ts*1R and $CVB3_m$ virus particles. This methodology did not distinguish between amyocarditic and myocarditic variants, since ^{125}I surface labeling of polypeptide patterns for *ts*1R and $CVB3_m$ were indistinguishable.

DISCUSSION

The isolation of revertant variants from stocks of *ts* mutant viruses derived from $CVB3_m$ was easily accomplished (39). Some of the revertants were myocarditic, whereas others resembled their immediate *ts* parent and were amyocarditic (39). In this study, an amyocarditic revertant and a myocarditic revertant were compared with the myocarditic parent in several properties which might be covariant with myocarditic capability. Major differences were not found in virion polypeptides by several techniques, which could account for the amyocarditic nature of *ts*1R compared with *ts*10R and $CVB3_m$. Only one minor difference in the surface region of a virion polypeptide was found among the three variants, and the small quantitative differ-

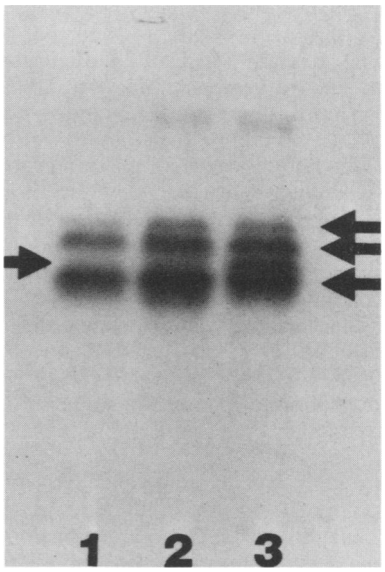

FIG. 4. Polyacrylamide gel electrophoresis of polypeptides from purified, surface-iodinated particles of *ts*10R, *ts*1R, and $CVB3_m$. Purified virus particles were iodinated, and the solubilized polypeptides were electrophoresed on a 12.5% polyacrylamide slab gel and processed for autoradiography as described in the text. Lanes 1, 2, and 3 represent electrophoretic profiles of surface-labeled polypeptides from *ts*10R, *ts*1R, and $CVB3_m$, respectively. The arrows on the right denote the positions of VP1, VP2a, and VP3, reading from top to bottom, respectively, and the arrow on the left denotes the position of VP2b.

ence was between *ts*10R and $CVB3_m$ in ^{125}I labeling of VP2b. Alterations in the genomes of *ts*1R, *ts*10R, and *ts*10 were found relative to the genome of $CVB3_m$ by oligonucleotide fingerprint mapping of T_1 RNase digest products. The number of changes found between two virus variants revealed that from one to seven oligonucleotides were different. These CVB3 variants could be distinguished from one another on a subgenomic basis, but differences in pathogenicity could not be associated with a particular oligonucleotide pattern obtained. Several variants of poliovirus serotypes 2 and 3, isolated from vaccinated patients with paralytic poliomyelitis, exhibited oligonucleotide maps similar to that of the vaccine strain, yet most isolates showed at least 1 (some showed up to 24) detectable changes in T_1-resistant oligonucleotides (24). Two isolates from neural tissues exhibited little to no changes in oligonucleotide maps when compared with the vaccine strains, suggesting that neurovirulence may be restored by a small number of genetic changes that may not be detected by this technique (24). The usefulness of the method in reproducibly disassembling a picornavirus genome was demonstrated for both poliovirus (24) and coxsackievirus B5 variants (12). Frisby et al. (12) found that this method could discriminate between two coxsackievirus B5 variants that were isolated 20 years apart. Trent et al. (37) used this method to demonstrate that antigenically related strains of Venezuelan equine encephalomyelitis exhibited similar oligonucleotide fingerprint patterns and that viruses within a dissimilar antigenic group exhibited a different pattern which was shared by all viruses within that group.

Susceptibility to viral infections has been reported to be associated with the major histocompatibility complex in mice (1, 5, 36, 41, 46, 47) and humans (4, 30, 40). However, Cambridge et al. (7) did not find a correlation between a specific *HLA* haplotype and congestive cardiomyopathy. Although it has been recognized that the strain of mouse used is important in determining the extent of myocarditis induced by CVB3 (44), our data showed that genetic background of the host, relative to CVB3 induction of myocarditis, is not a one- or two-gene phenomenon. It was shown that mouse strains sharing the same *H-2* haplotype were either highly susceptible or highly resistant to the induction of myocarditis. This finding suggests that a factor(s) outside of the major histocompatibility and *Lyt* loci is important in determining susceptibility to the induction of myocarditis.

The gender of a mouse was previously thought to be a viral myocarditis susceptibility factor (44). In earlier studies performed with a limited number of strains, males were consistently susceptible, whereas females were consistently resistant to the induction of myocarditis by the group B coxsackieviruses (44). Our findings do not agree with such a sex-related correlation. Male mice of some strains did exhibit greater susceptibility than females, but in other strains, the reverse was found, and in other strains, no differences were found. When gender was an important factor in determining susceptibility, it exerted a strong influence: male 129/J mice averaged 29.1 lesions per section, and female mice showed only 1.1 lesions per section in response to $CVB3_m$, whereas CBA/T6J mice showed an inverse pattern, with females developing an average of 35.8 lesions per section and males averaging only 1.7 lesions per section. From these findings, it appears that some factor(s) associated with the gender of the animals serves to modulate an initial state of susceptibility determined by other factors.

In previous studies (44) in which male mice developed more severe $CVB3_m$-induced myocarditis than did female mice, male mice possessed splenic T lymphocytes capable of lysing virus-infected syngeneic target cells (42), whereas splenic lymphocytes from virus-inoculated female mice had weak or no cytotoxic T-cell activity against infected target cells (20, 21, 43, 44). In contrast, female mice inoculated with $CVB3_m$ exhibited far greater NK-cell reactivity against several uninfected target cells than did virus-inoculated male mice (19). Cohn (10) found that low immune performance, i.e., specific-antibody synthesis, in male mice correlated well with mouse strains which exhibited high target organ responsiveness to androgen, although he found that sex differences in the immune response did not invariably occur. Unfortunately, BALB/c and CBA/J mice, which were used in the studies of sex differences in CVB3-induced myocarditis (20, 21, 43), were not included in the studies by Cohn (10). It has been well established that, in humans, CVB3-induced myocarditis is more severe in males than in females (18, 29).

For phenotypic expression (myocarditis) of both $CVB3_m$ and *ts*10R, *H-2* and *Lyt* genes clearly affected the severity of myocarditis induced. At present, we can only speculate on how these genes might be important to the induction of myocarditis. The *Lyt* genes are associated with surface antigen found on the surface of T lymphocytes, where they serve to identify functionally different subsets of T cells (9). At present, it is not clear whether *Lyt* loci are associated with any disease process or whether allelic differences at any of the *Lyt* loci are associated with functional differences in *Lyt*-bearing cells. How the *H-2* locus might be important in induction and expression of CVB3-induced myocarditis is presently unclear. Our findings do not suggest the presence of a major virus susceptibility gene for CVB3 within the *H-2* locus. This is because selection of mice with specific *H-2* genotypes did not reveal an all-or-none response or show major quantifiable changes in response to CVB3, as measured by the induction of myocarditis. Because the *H-2* locus in mice contains the *I* region (3, 48), which appears to control the function of T-cell subsets, it is possible that virus-induced changes in expression of the *I* region may act synergistically with changes in the *Lyt* region to alter T-cell functions in response to the myocarditic capabilities of CVB3. Surface changes induced by CVB3 in infected cells are currently under study, and these changes may be associated with *H-2* surface antigens, as has been reported for other viruses (46, 48).

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LITERATURE CITED

1. **Bank, G. B.** 1978. Genetics of resistance of animals to viruses. I. Introduction and studies in mice. Adv. Virus Res. **23**:269–348.
2. **Beatrice, S. T., M. G. Katze, B. A. Zajac, and R. L. Crowell.** 1980. Induction of neutralizing antibodies by the coxsackievirus B3 virion polypeptide, VP2. Virology **104**:426–438.
3. **Benacerraf, B.** 1981. Role of MHC gene products in immune regulation. Science **212**:1229–1238.
4. **Bertrams, J.** 1978. HLA and disease association. Behring Inst. Mitt. **62**:69–92.
5. **Braciale, T. J., G. L. Ada, and K. L. Yap.** 1978. Functional and structural consideration in the recognition of virus-infected cells by cytotoxic T lymphocytes. Contemp. Top. Mol. Immunol. **7**:319–364.
6. **Burch, G. E., and T. D. Giles.** 1972. The role of viruses in the production of heart disease. Am. J. Cardiol. **29**:231–240.
7. **Cambridge, G., C. G. C. MacArthur, A. Waterson, J. F. Goodwin, and C. M. Oakley.** 1979. Antibodies to coxsackie B viruses

in congestive cardiomyopathy. Br. Heart J. **41**:692–696.
8. **Campbell, A. E., R. M. Loria, and G. E. Madge.** 1978, Coxsackievirus B cardiopathy and angiopathy in the hypercholesterolemic host. Atherosclerosis **31**:295–306.
9. **Cantor, H., and E. A. Boyse.** 1975. Functional subclasses of T lymphocytes bearing different Lyt antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J. Exp. Med. **141**:1376–1399.
10. **Cohn, D. A.** 1979. High sensitivity to androgen as a contributing factor in sex differences in the immune response. Arthritis Rheum. **22**:1218–1231.
11. **Feinstone, S. M., T. Hensley, and M. W. Rytel.** 1973. Postcoxsackievirus B3 myocardiopathy in mice. Proc. Soc. Exp. Biol. Med. **144**:345–350.
12. **Frisby, D. P., C. Newton, N. H. Carey, P. Fellner, J. G. E. Newman, T. J. R. Harris, and F. Brown.** 1976. Oligonucleotide mapping of picornavirus RNAs by two-dimensional electrophoresis. Virology **71**:379–388.
13. **Garvey, J., N. E. Cremer, and D. H. Sussdorf.** 1981. Methods in immunology. A laboratory text for instruction and research, 3rd ed., p. 178–182. The Benjamin/Cummings Publishing Co., Reading, Mass.
14. **Gauntt, C. J., M. D. Trousdale, D. R. L. LaBadie, R. E. Paque, and T. Nealon.** 1979. Properties of coxsackievirus B3 variants which are amyocarditic or myocarditic for mice. J. Med. Virol. **3**:207–220.
15. **Gauntt, C. J., M. D. Trousdale, J. C. Lee, and R. E. Paque.** 1983. Preliminary characterization of coxsackievirus B3 temperature-sensitive mutants. J. Virol. **45**:1037–1047.
16. **Gudvangen, R. J., P. S. Duffey, R. E. Paque, and C. J. Gauntt.** 1983. Levamisole exacerbates coxsackievirus B3-induced murine myocarditis. Infect. Immun. **41**:1157–1165.
17. **Habel, K.** 1969. Virus neutralization test, p. 294–295. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press, Inc., New York.
18. **Hirschman, S. Z., and G. S. Hammer.** 1974. Coxsackie virus myopericarditis: a microbiological and clinical review. Am. J. Cardiol. **34**:224–232.
19. **Huber, S. A., L. P. Job, K. R. Auld, and J. F. Woodruff.** 1981. Sex-related differences in the rapid production of cytotoxic spleen cells active against uninfected myofibers during coxsackievirus B-3 infection. J. Immunol. **126**:1336–1340.
20. **Huber, S. A., L. P. Job, and J. F. Woodruff.** 1980. Lysis of infected myofibers by coxsackievirus B-3-immune T lymphocytes. Am. J. Pathol. **98**:681–694.
21. **Huber, S. A., L. P. Job, and J. F. Woodruff.** 1981. Sex-related differences in the pattern of coxsackievirus B-3-induced immune spleen cell cytotoxicity against virus-infected myofibers. Infect. Immun. **32**:68–73.
22. **Katze, M. G., and R. L. Crowell.** 1980. Immunological studies of the group B coxsackievirus by the sandwich enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. J. Gen. Virol. **50**:357–367.
23. **Kawai, C., A. Matsumori, N. Kumagai, and M. Tokuda.** 1978. Experimental coxsackie virus B-3 and B-4 myocarditis in mice. Jpn. Circ. J. **42**:43–47.
24. **Kew, O. M., B. K. Nottay, M. H. Hatch, J. H. Nakano, and J. F. Objeski.** 1981. Multiple genetic changes can occur in the oral poliovaccines upon replication in humans. J. Gen. Virol. **56**:337–347.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Lansdown, A. B. G.** 1978. Viral infections and diseases of the heart. Med. Virol. **24**:70–113.
27. **Lerner, A. M., and F. M. Wilson.** 1973. Virus myocardiopathy. Prog. Med. Virol. **15**:63–91.
28. **Levine, H. D.** 1979. Virus myocarditis: a critique of the literature from clinical, electrocardiographic, and pathologic standpoints. Am. J. Med. Sci. **277**:132–144.
29. **Morens, D. M.** 1978. Enteroviral disease in early infancy. J. Pediatr. **92**:374–377.
30. **Morris, P. J., and M. C. Pietsch.** 1973. A possible association between paralytic poliomyelitis and multiple sclerosis. Lancet **ii**:847–848.
31. **Paque, R. E., and C. J. Gauntt.** 1983. Cellular and humoral immune responses in the induction and expression of viral-induced myocarditis, p. 47–69. *In* J. A. Robinson and J. O'Connell (ed.), Myocarditis: a precursor to cardiopathy. Collamore Press, D.C. Heath Co., Lexington, Mass.
32. **Paque, R. E., C. J. Gauntt, and T. J. Nealon.** 1981. Assessment of cell-mediated immunity against coxsackievirus B3-induced myocarditis in a primate model (*Papio papio*). Infect. Immun. **31**:470–479.
33. **Paque, R. E., C. J. Gauntt, T. J. Nealon, and M. D. Trousdale.** 1978. Assessment of cell-mediated hypersensitivity against coxsackievirus B3 viral-induced myocarditis utilizing hypertonic salt extracts of cardiac tissue. J. Immunol. **120**:1672–1678.
34. **Roesing, T. G., B. J. Landau, and R. L. Crowell.** 1979. Limited persistence of viral antigen in coxsackievirus B3 induced heart disease in mice. Proc. Soc. Exp. Biol. Med. **160**:382–386.
35. **Rytel, M. W., and E. D. Kilbourne.** 1971. Differing susceptibility of adolescent and adult mice to non-lethal infection with coxsackievirus B3. Proc. Soc. Exp. Biol. Med. **137**:443–448.
36. **Swierkosz, J. E., P. Marrack, and J. W. Kappler.** 1979. The role of H-2-linked genes in helper T cell function V. I. Region control of helper T cell interaction with antigen-presenting macrophages. J. Immunol. **123**:654–659.
37. **Trent, D. W., J. P. Clewley, J. K. France, and D. H. L. Bishop.** 1979. Immunochemical and oligonucleotide fingerprint analyses of Venezuelan equine encephalomyelitis complex virus. J. Gen. Virol. **43**:365–381.
38. **Trousdale, M. D., R. E. Paque, and C. J. Gauntt.** 1977. Isolation of coxsackievirus B3 temperature-sensitive mutants and their assignment to complementation groups. Biochem. Biophys. Res. Commun. **76**:368–375.
39. **Trousdale, M. D., R. E. Paque, T. Nealon, and C. J. Gauntt.** 1979. Assessment of coxsackievirus B3 *ts* mutants for induction of myocarditis in a murine model. Infect. Immun. **23**:486–495.
40. **Ward, C., K. Gelsthorpe, and R. W. Doughty.** 1976. A relation between HLA antigens and clinical features in patients with acquired valvular heart disease. Br. Med. J. **1**:1499–1501.
41. **Webb, S. R., and G. E. Madge.** 1980. The role of host genetics in the pathogenesis of coxsackievirus infection in the pancreas of mice. J. Infect. Dis. **141**:47–54.
42. **Wong, C. Y., J. J. Woodruff, and J. F. Woodruff.** 1977. Generation of cytotoxic T lymphocytes during coxsackievirus B-3 infection. II. Characterization of effector cells and demonstration of cytotoxicity against viral-infected myofibers. J. Immunol. **118**:1165–1169.
43. **Wong, C. Y., J. J. Woodruff, and J. F. Woodruff.** 1977. Generation of cytotoxic T lymphocytes during coxsackievirus B-3 infection. III. Role of sex. J. Immunol. **119**:591–597.
44. **Woodruff, J. F.** 1980. Viral myocarditis. A review. Am. J. Pathol. **101**:425–483.
45. **Woodruff, J. F., and J. J. Woodruff.** 1974. Involvement of T lymphocytes in the pathogenesis of coxsackievirus B3 heart disease. J. Immunol. **113**:1726–1734.
46. **Zinkernagel, R. M., and P. C. Doherty.** 1976. The role of major histocompatibility antigens in cell-mediated immunity to virus infections, p. 735–750. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), Animal virology. Academic Press, Inc., New York.
47. **Zinkernagel, R. M., and P. C. Doherty.** 1977. Major transplantation antigens, viruses, and specificity of surveillance T cells. Contemp. Top. Immunobiol. **7**:179–220.
48. **Zinkernagel, R. M., M. B. C. Dunlop, R. V. Blanden, P. C. Doherty, and D. C. Shreffler.** 1976. H-2 compatibility requirement for virus-specific T-cell mediated cytolysis. Evaluation of the role of H-2 I region and non H-2 genes in regulating the immune response. J. Exp. Med. **144**:519–532.