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Temperature-Sensitive Mutant of Coxsackievirus B3 Establishes Resistance in Neonatal Mice That Protects Them During Adolescence Against Coxsackievirus B3-Induced Myocarditis

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Inoculation of neonatal CD-1 mice by multiple routes with an amyocarditic temperature-sensitive (*ts*) mutant (*ts* 1) derived from a myocarditic parent variant of coxsackievirus B3 ($CVB3_m$) resulted in approximately half of the neonates surviving to adolescence. Challenge of the *ts* 1 survivors with $CVB3_m$ did not induce myocarditis, as assessed by histological examination of heart tissues. Virus was not detected in heart tissues of adolescent *ts* 1 survivors, but inoculation of these mice with $CVB3_m$ resulted in virus concentrations similar in titers to those found in $CVB3_m$-inoculated normal adolescent mice. The *ts* 1 survivors did not contain detectable levels of anti-$CVB3_m$ neutralizing antibody, but upon challenge with $CVB3_m$ they produced antibody more rapidly and to higher titers than did normal CD-1 adolescents after primary inoculation with $CVB3_m$. Cell-mediated immunity in *ts* 1 survivors was compared with that of normal mice after challenge with $CVB3_m$. The capacity for production of migration inhibitory factor was assessed by the agarose droplet cell migration inhibition assay, using peritoneal exudate cells and a $CVB3_m$ cell lysate or KCl-extracted antigens from heart tissues of $CVB3_m$-inoculated mice. Migration inhibitory factor activity was not detected in cultures of splenic leukocytes from *ts* 1 survivors of $CVB3_m$-inoculated *ts* 1 survivors, but it was readily detected in cultures of splenic leukocytes from $CVB3_m$-inoculated normal adolescent mice. The [^{3}H]thymidine stimulation assay, performed with splenic lymphoid cells and purified $CVB3_m$ particles, revealed that lymphocytes from normal mice, whether inoculated with $CVB3_m$ or not, were not stimulated by $CVB3_m$ particle antigens, whereas lymphoid cells from a significantly higher proportion of *ts* 1 survivors, whether inoculated with $CVB3_m$ or not, responded with a stimulation index ≥ 2.0. The cells responding with positive stimulation were T lymphocytes. A higher proportion of normal mice and *ts* 1 survivors, both inoculated with $CVB3_m$, contained splenic cytotoxic T lymphocytes with higher reactivity against $CVB3_m$-infected neonatal skin fibroblasts than against normal skin fibroblasts, as assessed by a ^{51}Cr release assay. The group of uninoculated *ts* 1 survivors present as a high proportion of individuals with cytotoxic T-lymphocyte reactivity against both uninoculated and $CVB3_m$-inoculated skin fibroblasts. However, *ts* 1 survivors and normal mice possessed the same proportions of splenic lymphocytes carrying either allele for Lyt 1 and Lyt 2 surface markers. The results suggest two mechanisms by which *ts* 1 survivors exhibit resistance to $CVB3_m$ induction of myocarditis, namely, the rapid production of high-titered anti-$CVB3_m$ neutralizing antibody in response to $CVB3_m$ inoculation and altered cell-mediated immune responses against $CVB3_m$-induced viral or novel cellular antigens. The data are compatible with the notion that an immune deviation mechanism, thought to be controlled through a mechanism requiring suppressor cell activity which inhibits macrophage activation in *ts* 1 survivors, protects these mice from induction of myocarditis.

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The murine coxsackievirus B (CVB) model of myocarditis is thought to approximate CVB-induced myocarditis in humans (5, 11, 25, 26, 42, 49). Infection of mice by several routes results in virus replication in the liver, spleen, pancreas, and heart. Virus titers in most organs generally fall below detectable levels within a week; the exceptions are the heart and pancreas, where virus may be found for an additional 3 to 7 days (24, 40). Despite the absence of infectious virus within the heart, this organ displays pathological lesions in the form of discrete localized foci of infiltrating mononuclear cells and necrotic myofibrils (15, 33, 47, 50, 56) which may persist for many weeks (24, 40, 56).

The mechanism of pathogenesis in CVB-induced murine myocarditis is unknown, although considerable evidence suggests that cell-mediated immunity or delayed hypersensitivity plays a role (15, 17, 21, 32, 33, 48, 51–53, 55, 56). In the coxsackievirus B3 (CVB3)-murine model, thymus-derived CVB3-sensitized T lymphocytes have been implicated in mediating cytotoxicity against virus-infected heart cells (21, 50–53). The nature of the antigen(s) in CVB3-infected heart tissues is of major importance in understanding the disease process. Paque et al. have shown that heart tissues from CVB3-infected mice (33, 34) or baboons (32) contain a KCl-extractable antigen(s) which reacts immunospecifically with CVB3-sensitized peritoneal exudate cells from CVB3-immunized or infected animals in the cell migration inhibition assay. This antigen(s), having a molecular weight of approximately 50,000, apparently does not cross-react with CVB3 virion antigens (33, 34). The genotype of the virus seems to be relevant to pathogenesis, since the immunoreactive antigen(s) was obtained only from heart tissues of mice inoculated with myocarditic but not amyocarditic variants of CVB3 (15, 48). This antigen(s) may represent a CVB3-induced nonvirion antigen or a CVB3-induced cellular neoantigen (33, 34, 40). Wong et al. (51) and Huber et al. (21) showed that cytotoxic T lymphocytes from CVB3-infected mice react against both uninfected and infected syngeneic fetal heart tissues, suggesting that CVB3-induced myocarditis represents, in part, a form of autoimmunity.

Previous studies (48) in our laboratories demonstrated that prototype strains of temperature-sensitive (*ts*) mutants of CVB3, representing each of the three nonoverlapping complementation groups (47), could serve as vaccine strains to protect adolescent CD-1 mice from myocarditis induced by the parental myocarditic CVB3 variant ($CVB3_m$). The three prototype *ts* variants retained some pathogenicity for neonates, as approximately half of the neonates inoculated with two prototype viruses (*ts* 1, ts 11) died before adolescence (48). In the present study, we report that challenge of *ts* 1 survivors with the parent myocarditic $CVB3_m$ variant did not result in induction of myocarditis. These resistant mice were then studied to assess the contribution of immune responses to CVB3-induced myocarditis. The results suggest that resistance to $CVB3_m$-induced myocarditis in the *ts* 1 survivor mice is due to two factors: (i) a rapid production of anti-$CVB3_m$ antibody to high titer, and (ii) altered T-cell responses to $CVB3_m$ antigens or to the $CVB3_m$-induced immunoreactive heart tissue antigen(s).

MATERIALS AND METHODS

Mice. Young breeding pairs of CD-1 mice were purchased from Charles River Breeding Laboratories, Inc., Boston, Mass., and maintained in the Laboratory Animal Resources facilities of The University of Texas Health Science Center at San Antonio. Adolescent CD-1 and BALB/c mice were purchased from Jackson Laboratories, Bar Harbor, Maine. All mice were given fresh water daily and placed on a standard diet of laboratory mouse chow ad libitum. Neonates less than 24 h old were inoculated (see below) with *ts* 1 virus to obtain *ts* 1 survivors. Adolescent mice 4 to 6 weeks of age (17 to 21 g) and of both sexes were used in all experiments, except for the cytotoxic T-lymphocyte assays, in which only male mice were used. Adult mice were used as the source of sensitized peritoneal exudate cells (PEC) for the cell migration inhibition assay as previously described (33).

Cell culture and media. HeLa cells used in this study were obtained from the American Type Culture Collection (ATCC CCL 2), and stocks were cultured in Auto-Pow minimum essential medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.5 μg of amphotericin B per ml, and 0.056% $NaHCO_3$. Other cell culture reagents were purchased from Grand Island Biological Co. (GIBCO), Grand Island, N.Y. Cells inoculated with viruses were incubated in virus growth medium (minimal essential medium containing 1% heat-inactivated fetal bovine serum and glutamine, antibiotics, and $NaHCO_3$ as described above).

Viruses. The origins, preparations of virus stocks, and plaque assay methods for $CVB3_m$ and for the (*ts*) mutants derived from this parent virus have been described previously (47). The *ts* 1 variant does not appear to replicate in heart tissues of adolescent mice and does not produce myocarditis in these mice (48). The parent $CVB3_m$ virus, however, produced readily detectable levels of myocarditis (see below) in 95% of adolescent CD-1 mice at intraperitoneal (i.p.) inoculum doses of 10^4 to 10^7 PFU per 0.2 ml at 8 days post-inoculation (p.i.). For comparative purposes with other data on this topic (51–54, 56), 10^7 tissue culture infectious doses of $CVB3_m$ per ml equals approximately 10^7 PFU/ml.

Assay of heart tissues for $CVB3_m$. The apical one-third portion of hearts from each group of mice were weighed, pooled in 1 or 2 ml of minimal essential medium, minced with scissors, and disrupted with 20

strokes of a tight-fitting Dounce homogenizer in an ice bath. The homogenates were frozen (−90°C) and thawed (37°C) three times, and the entire suspension was plaque assayed for total infectious virus.

Virus inoculation and immunization of mice. The *ts* 1 survivors used in this study were obtained after inoculation of neonatal mice by intracerebral, subcutaneous, and i.p. routes with 0.02 to 0.05 ml of a stock virus solution containing 10^7 PFU/ml. The newborn mice were observed daily for mortality. Survival to adolescence of *ts* 1 virus-inoculated neonates, hereafter denoted *ts* 1 survivors, was approximately 52% (453 of 866). There was no effect of litter size (5 to 8, 9 to 13, or ≥14) on the proportion of mice surviving *ts* 1 virus inoculation, as assessed by chi-square analysis ($P < 0.05$) of mice in 56 litters. Adult mice which were used as the source of PEC for the cell migration inhibition assay were immunized via the i.p. route with a solution containing the equivalent of 10^8 PFU of UV-radiation-inactivated virus in 0.1 ml of Hanks balanced salt solution and an equal volume of Freund complete adjuvant containing 500 μg of *Mycobacterium butyricum* per ml (Difco Laboratories, Detroit, Mich.) (33). UV irradiation of virus was carried out with a 150-W germicidal lamp (General Electric Co., Schenectady, N.Y.) at a distance of 12 cm for 5 min, resulting in inactivation of stocks from 1×10^9 or 2×10^9 to $<10^2$ PFU/ml. Two weeks after initial inoculation, the animals were boosted by i.p. inoculation of 0.1 ml of 10^8 PFU of inactivated virus. Mice from which hearts were to be obtained for KCl extraction (33, 48) were inoculated i.p. with 10^8 PFU per 0.1 ml of infectious $CVB3_m$; 8 days after inoculation, the mice were sacrificed.

Histology. Hearts were removed and fixed in 10% Formalin (diluted in phosphate-buffered saline, pH 7.4) for 24 h, embedded in paraffin, and stained with hematoxylin and eosin (33). A minimum of two to four sections per heart were examined for myocardial lesions, defined as focal, irregularly shaped areas containing myocytes undergoing necrosis, with interstitial spaces infiltrated with numerous mononuclear and polymorphonuclear leukocytes (56). Myocarditis was scored per mouse or group of mice according to a previously described scale (48): 0 (no lesions); ± (1 to 2 lesions per section); 1+ (3 to 7 lesions per section); 2+ (8 to 20 lesions per section); 3+ (21 to 50 lesions per section); and 4+ (>50 lesions per section).

Cell migration inhibition assay. The agarose droplet cell migration inhibition assay, as described by Harrington and Stastny (16) and modified by Paque et al. (33, 34), was employed as an in vitro correlate of cellular immunity. The heart tissues were extracted with hypertonic 3 M KCl according to Reisfeld and Kahan (39) with the modifications of Meltzer et al. (27), except that heart tissues were disrupted by three 1-min shearings at 0°C with a Lourdes Omnimixer at a maximum setting. Protein concentrations in the antigen preparations were measured by using the dye-binding assay of Bradford (4; Bio-Rad Laboratories, Richmond, Calif.). In calculations of the cell migration inhibition results, a mean migration index (MMI) of ≤65% was considered positive inhibition of macrophage migration, based on previous statistical analyses of data from this assay (2).

Determination of serum antibody and interferon titers by plaque reduction assays. Serum antibody titers to $CVB3_m$ were determined by a plaque reduction assay in HeLa cells, as previously described (15). At endpoint, the reciprocal of that dilution of serum which resulted in a 90% or greater reduction in numbers of 1,000 PFU of $CVB3_m$ was taken as the titer. Serum interferon titers were measured by a 50% reduction in titer of 50 to 100 PFU of vesicular stomatitis virus plaques, as previously described (15), except that sera were not adjusted to pH 2 before assay. Mouse reference standard interferon was obtained from the National Institutes of Health, National Institute of Allergy and Infectious Diseases Reference Reagent Branch.

[^{3}H]thymidine stimulation test with splenic lymphoid cells. Splenic lymphoid cells were obtained from *ts* 1 survivors and normal mice which were sacrificed by cervical dislocation. Spleens were removed aseptically, and cells were expressed from the spleens with a rubber policeman into phosphate-buffered saline, pH 7.2. After vigorous pipetting and two washes with phosphate-buffered saline, one part of the cell suspension was mixed with four parts of 0.15 M NH_4Cl-Tris buffer (3) and incubated at 37°C for 10 min to lyse erythrocytes. The cells were then mixed with an equal volume of phosphate-buffered saline and centrifuged at 350 × *g* for 10 min. After two washes with RPMI 1640 medium, the cells were counted and diluted in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (56°C for 30 min), 100 μg of streptomycin per ml, and 100 U of penicillin per ml. Total and viable counts were performed by nigrosin dye exclusion (13). Cells were placed in microcultures according to a modification of the procedure of Janossy and Greaves (22). Purified $CVB3_m$ particles for use as antigens in this test were prepared by a method previously used for rhinovirus type 14 (14). Optimum test parameters employed 5×10^5 cells per well in a total volume of 0.15 ml, including 0.1 ml of purified $CVB3_m$ preparation (10^7 PFU) and [^{3}H]thymidine (6 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) added to a final concentration of 1 μCi/ml on day 3 after establishing the cell culture. All cultures were set up in triplicate, harvested on day 4, collected, and processed, using a multiple automated sample harvester (MASH II, Microbiological Associates, Bethesda, Md.). Each filter disk was incubated in 0.3 ml of Protosol (New England Nuclear Corp., Boston, Mass.) at 50°C for 2 h before the addition of toluene-Liquifluor (New England Nuclear) scintillation fluid. Radioactivity was measured in a Mark III 6800 liquid scintillation spectrometer (Searle Analytic, Inc., Des Plaines, Ill.).

Cytotoxic lymphocyte assay. The technique for the cytotoxic lymphocyte assay was that of Wong et al. (51, 52). Skin fibroblasts were prepared from CD-1 neonates <24 h of age as previously described (51). After culture for 3 to 8 days at 37°C in CMRL 1066 medium or RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics, the cells were seeded at 3×10^3 to 5×10^3 cells per well in a 96-well flat bottom MicroTest II tissue culture plate (Falcon Plastics, Oxnard, Calif.). After 2 to 20 h of additional incubation, half of the cell cultures were challenged with 75 to 150 PFU per cell. Control uninfected cells received medium only. On day 3 p.i., all cell cultures were washed and incubated in 0.1 ml of one or the other growth medium cited above containing ^{51}Cr at 50

μCi/ml for 4 h at 37°C. At the end of this incubation period, the radiolabeled medium was removed, and the cell cultures were washed three times in either growth medium. Male mice were sacrificed at 7 days p.i., and splenic lymphoid cells were incubated for 45 min at 37°C to remove adherent cells. Nonadherent cells were added to skin fibroblast cultures at a 100 to 150:1 effector-to-target cell ratio and incubated for 18 to 20 h at 37°C. All tests were performed at least in triplicate, and most were done in sextuplicate.

Treatment of nonadherent cells with anti-theta antiserum and complement. Approximately 1×10^7 to 5×10^7 nonadherent splenic lymphocytes per ml were incubated with 40 μl of anti-mouse T cell serum (Cederlane Laboratories Ltd., London, Canada) for 30 min in an ice bath. After the cells were washed twice, complement (Low-Tox-M rabbit complement, Cederlane Laboratories, Ltd.) was added at 1 part complement:3 parts cells, and the whole was incubated at 37°C for 30 min. These cells were then washed twice and diluted into an appropriate medium.

Statistics. Standard one-way analysis of variance, Student's two tailed *t* test, and chi-square analyses with Yates correction factor were performed on a DATA GENERAL NOVA computer.

TABLE 1. Resistance to $CVB3_m$ induction of myocarditis in adolescent *ts* 1 survivors[a]

Expt no.	Murine group	Challenge with $CVB3_m$	Avg no. of lesions per heart section[b]	Lesion score[d]
1	Normals	−	0	0
	Normals	+	9.8 ± 5.4[c]	2+
	Ts 1 survivors	−	0	0
	Ts 1 survivors	+	0.3 ± 0.5[c]	0
2	Normals	−	0	0
	Normals	+	36.0 ± 13.2[c]	3+
	Ts 1 survivors	−	0	0
	Ts 1 survivors	+	0[c]	0

[a] Adolescent mice (4 to 6 weeks) in groups of 10 to 15 were challenged i.p. with 10^7 PFU of virus. Mice were sacrificed at 8 days p.i., and hearts were fixed in Formalin.

[b] Two sections of each heart were examined for myocarditic lesions, areas of mononuclear cell infiltration, and necrotic cells. Values indicate mean ± standard deviation.

[c] $P < 0.001$ by *t* test.

[d] Scoring system (scores represent average number of lesions per section): 0, no lesions; ±, 1 to 2; 1+, 3 to 7; 2+, 8 to 20; 3+, 21 to 50; 4+, >50.

RESULTS

Susceptibility of *ts* 1 survivors to induction of myocarditis by $CVB3_m$. Heart tissues from adolescents surviving inoculation with *ts* 1 virus by multiple routes as neonates exhibited no lesions (Table 1), and sections from these hearts could not be distinguished in appearance from sections of normal control hearts. Subsequent inoculation of *ts* 1 survivors with the myocarditic parent strain, $CVB3_m$, resulted in induction of minimal to no myocarditis. However, normal adolescent mice inoculated with $CVB3_m$ displayed obvious evidence of myocarditis. Late in the study, we found that i.p. inoculation of neonates with *ts* 1 at the same concentration rendered the survivors (95 to 100%) similarly resistant to induction of myocarditis by $CVB3_m$ during adolescence (C. J. Gauntt, unpublished data). However, all studies described hereafter were conducted with *ts* 1 survivors which had been inoculated by multiple routes (i.p., subcutaneously, and intracerebrally).

Replication of $CVB3_m$ in heart tissues of *ts* 1 survivors after challenge with $CVB3_m$ as adolescents. The lack of myocarditis in *ts* 1 survivors challenged with $CVB3_m$ as adolescents could be explained on the basis of an inability of $CVB3_m$ to replicate in heart tissues of survivors. This was clearly not the case (Table 2), as heart tissues of $CVB3_m$-inoculated *ts* 1 survivors contained levels of infectious $CVB3_m$ similar to those in $CVB3_m$-inoculated normal adolescent CD-1 mice at 8 days p.i. As in normal mice inoculated with $CVB3_m$, heart tissues from *ts* 1 survivors challenged with $CVB3_m$ do not contain detectable levels of $CVB3_m$ at 12 to 14 days p.i. (data not shown). Heart tissues from *ts* 1 survivors not challenged with $CVB3_m$ did not contain detectable infectious coxsackievirus B3 (*ts* 1 mutant virus) at 8 days p.i. Our experience is that approximately 250 PFU per g of tissue is the lower limit of the plaque assay under these conditions; variability in the lower limit shown for the three experiments is due to a lack of detection of any virus in different quantities of heart tissues taken for assay.

Levels of $CVB3_m$-neutralizing antibody in the sera of adolescent CD-1 mice surviving inoculation of *ts* 1 virus. An obvious mechanism of resistance to challenge with $CVB3_m$ would be the production of neutralizing antibody to $CVB3_m$. Accordingly, serum antibody titers to $CVB3_m$ were assessed in $CVB3_m$-challenged normal mice and *ts* 1 survivors at 8 days p.i. by a plaque-reduction technique (Table 3). Adolescent *ts* 1 survivors not challenged with $CVB3_m$ did not have detectable levels of virus-neutralizing antibody in their serum. We do not know whether *ts* 1 survivors had nonneutralizing serum antibodies directed toward $CVB3_m$ particle antigens; had we used a highly sensitive enzyme-linked immunosorbent assay technique (23), we might have detected such antibodies. Subsequent i.p. challenge of *ts* 1 survivors with $CVB3_m$ resulted in production of high titers of anti-$CVB3_m$ neutralizing antibody. Seven of eight *ts* 1 survivors challenged with $CVB3_m$ exhibited anti-$CVB3_m$ neutralizing antibody ti-

TABLE 2. Virus contents of heart tissues taken from *ts* 1 survivors after challenge with $CVB3_m$[a]

Expt	Experimental group	Challenge with $CVB3_m$[b]	Titer (PFU per g of tissue 8 days p.i.)	Lesion score[c] (avg no. of lesions per heart section)
1	*Ts* 1	No	$<3.0 \times 10^2$	0 (0)
	Ts 1	Yes	1.2×10^5	0 (0)[d]
	Normals	Yes	1.2×10^5	3+ (36.0 ± 21.1)[d]
2	*Ts* 1	No	$<1.2 \times 10^3$	0 (0)
	Ts 1	Yes	1.0×10^5	0 (0)[d]
	Normals	Yes	2.1×10^4	1+ (3.5 ± 2.1)[d]
3	*Ts* 1	No	$<2.6 \times 10^2$	0 (0)
	Ts 1	Yes	5.6×10^4	0 (0.2 ± 0.4)[d]
	Normals	Yes	6.1×10^4	2+ (18.6 ± 9.5)[d]

[a] Heart tissues were harvested at 8 days p.i., Dounce homogenized, freeze-thawed three times, and sonicated, and virus contents were assessed by plaque assay.

[b] Mice (six per group) were challenged i.p. with 10^7 PFU.

[c] Lesion score and average number of lesions per heart section were determined as described in the footnotes to Table 1. Values indicate mean ± standard deviation.

[d] $P < 0.01$ by Student's *t* test.

ters at 8 days p.i., two- to five-fold higher than the highest titer produced in adolescent normal control mice challenged with $CVB3_m$.

Temporal production of neutralizing antibody to $CVB3_m$ was measured in the serum of $CVB3_m$-challenged *ts* 1 survivors and $CVB3_m$-challenged normal adolescent mice. The sera were obtained by serial retroorbital bleedings of six animals at 0, 3, 6, and 9 days p.i. (Table 4). Five of six *ts* 1 survivors challenged with $CVB3_m$ produced detectable levels of neutralizing antibody to $CVB3_m$ by 3 days p.i. (10 to 20 U), and the titers increased over the next 6 days. In contrast, normal adolescent CD-1 mice did not produce detectable levels of neutralizing antibody to $CVB3_m$ until day 6 p.i. Titers of neutralizing antibody to $CVB3_m$ at day 9 p.i. were much higher in $CVB3_m$-challenged *ts* 1 survivors than in most normal mice at this time. These results suggest that *ts* 1 survivors experienced an anamnestic response upon exposure to $CVB3_m$ particle antigens.

Immune reactivity of PEC from adolescent *ts* 1 survivors and $CVB3_m$-challenged *ts* 1 survivors tested against KCl-extracted antigens from heart tissues of $CVB3_m$-inoculated mice and against $CVB3_m$ HeLa cell lysate antigens. We have previously shown (15, 33, 34, 47) that heart tissues from adolescent mice inoculated with $CVB3_m$ contain KCl-extractable antigens which react specifically with $CVB3_m$-sensitized PEC obtained from normal CD-1 mice in the agarose droplet cell migration inhibition assay. PEC from $CVB3_m$-sensitized mice also react specifi-

TABLE 3. Production of serum-neutralizing antibody to $CVB3_m$ by *ts* 1 survivors subsequently challenged with $CVB3_m$

Source of sera	Challenge with $CVB3_m$	Antibody titers[b] from individual mice	Geometric mean titer	Lesion score (avg no. of lesions per heart section)[c–f]
Normal	No	<10, <10, <10, <10	<10	0 (0)
	Yes	20, 40, 20, 20 80, 40, 40, 20	29	2+ (18.4 ± 8.8)[d]
Ts 1 Survivors	No	<10, <10, <10, <10 <10, <10, <10, <10	<10	0 (0.3 ± 0.4)[e]
	Yes	640, 320, 640, 160 320, 40, 2560, 640	381	0 (0.3 ± 0.4)[f]

[a] Sera were taken at 8 days after challenge inoculation with $CVB3_m$.

[b] Antibody titer endpoints were the reciprocal of the antibody dilution which reduced the number of $CVB3_m$ plaques by 90% or greater in a HeLa cell plaque assay. By *t* test of mean antibody titers in $CVB3_m$-challenged normal mice versus mean antibody titers in $CVB3_m$-challenged *ts* 1 survivors, $P > 0.2$.

[c–f] Lesion score and average number of lesions per heart section were determined as described in the footnotes to Table 1. A *t* test showed the average number of lesions for *d* versus *e* or *f* was significantly different ($P < 0.01$).

TABLE 4. Temporal production of neutralizing antibody to CVB3 in the serum of normal and *ts* 1 survivors after challenge with $CVB3_m$[a]

Subject no.	Neutralizing antibody titers to $CVB3_m$[b]				Lesion score[c] (avg no. of lesions per heart section)
	Pre-bled	3 days p.i.	6 days p.i.	9 days p.i.	
N (1)	<10	<10	40	80	1+ (2.8)
N (2)	<10	<10	40	40	1+ (5.7)
N (3)	<10	<10	40	80	1+ (4.9)
N (4)	<10	<10	40	40	2+ (11.4)
N (5)	<10	<10	40	40	3+ (32.0)
N (6)	<10	<10	40	80	1+ (5.4)
(Geometric mean)		ND[d]	(40)	(57)	
Ts 1 (1)	<10	10	640	1,280	0 (0)
Ts 1 (2)	<10	20	80	80	0 (0)
Ts 1 (3)	<10	10	640	2,560	0 (0)
Ts 1 (4)	<10	20	80	320	0 (0)
Ts 1 (5)	<10	<10	40	320	0 (0)
Ts 1 (6)	<10	20	640	1,280	0 (0)
(Geometric mean)		ND	(199)	(570)	

[a] Adolescent mice were inoculated by the i.p. route with 10^7 PFU.

[b] Antibody titers were assessed as described in footnote *b* to Table 3. Antibody titers on day 9 of normal mice versus *ts* 1 survivors were significantly different ($P < 0.2$) by Student's *t* test.

[c] Lesion score and average number of lesions per heart section were determined as described in the footnotes to Table 1. Two sections were examined per heart in this experiment, and myocarditic scores from days 6 and 9 were averaged.

[d] ND, Not determined.

cally with $CVB3_m$ antigens in HeLa cell lysates containing at least 10^8 PFU per well (33). Using these two antigen preparations, we compared the immune reactivities of PEC from groups of uninoculated normal, $CVB3_m$-inoculated normal, *ts* 1 survivor, and $CVB3_m$-challenged *ts* 1 survivor mice; the results are shown in Table 5. All antigen preparations were titrated and tested for toxicity on normal PEC before the assays and found to be nontoxic (33). Controls in these tests confirmed previously published data (15, 33, 47), i.e., PEC from normal uninoculated adolescents did not react with antigens in KCl extracts of normal mouse hearts (MMI of 101 to 102), KCl extracts of hearts of $CVB3_m$-inoculated mice (MMI of 99 to 102), or a $CVB3_m$ cell lysate (MMI of 101). Also, PEC from $CVB3_m$-inoculated normal mice exhibited specific inhibition of migration in the presence of 200 to 400 μg of KCl extract of hearts from $CVB3_m$-inoculated mice (MMI of 45 to 61) and a $CVB3_m$ lysate (MMI of 35), but not in the presence of a KCl extract of normal hearts (MMI of 100 to 103). The PEC from *ts* 1 survivors not challenged with $CVB3_m$ did not react with antigens in KCl extracts of either source of heart tissues (MMI of 96 to 102) or the $CVB3_m$ lysate (MMI of 95 to 98), and neither did PEC from $CVB3_m$-inoculated *ts* 1 survivors react with antigens in KCl extracts of either source of heart tissues (MMI of 98 to 105). Surprisingly, the latter PEC also failed to react with the $CVB3_m$ cell lysate (MMI of 90 to 100); this was unexpected because $CVB3_m$ titers in heart tissues of these mice were similar to those of $CVB3_m$-inoculated normal adolescents (Table 2). These data suggested that cell-mediated immune responses to $CVB3_m$ infection in *ts* 1 survivors were different from those of normal mice. Accordingly, we employed a second method for assessing cell-mediated immune responses to $CVB3_m$ infections in *ts* 1 survivors and normal mice.

Stimulation indices in the [^{3}H]thymidine incorporation assay of splenic lymphoid cells from *ts* 1 survivors and $CVB3_m$-inoculated *ts* 1 survivors cultured with purified $CVB3_m$ virions. Spleen cells from individual mice in each of the four groups of animals (normals, $CVB3_m$-inoculated normals, *ts* 1 survivors, and $CVB3_m$-inoculated *ts* 1 survivors) were obtained at 8 days p.i. and placed in microculture with purified $CVB3_m$ virus particles. After incubation for 3 days, [^{3}H]thymidine was added, and the stimulation indices (SI) were calculated for each mouse. The results (Table 6) show that splenic lymphoid cells from only 1 of 14 normal mice and only 4 of 14 $CVB3_m$-inoculated normal mice gave positive responses (SI >2.0) to antigens in purified $CVB3_m$ particles. In contrast, splenic lymphoid cells from 6 of 13 *ts* 1 survivors and 6 of 13 $CVB3_m$-inoculated *ts* 1 survivors responded positively (SI >2.0) to purified $CVB3_m$ particle antigens and, in general, SI values were higher. The type of cells responding in this assay were

TABLE 5. Cell migration inhibition assay of PEC from *ts* 1 survivors and $CVB3_m$-inoculated *ts* 1 survivors against KCl-extracted heart tissue antigens from normal and $CVB3_m$-inoculated mice and a $CVB3_m$ HeLa cell lysate[a]

Source of PEC	Source of heart tissue antigens[b] (μg/well) or [PFU/well]	Expt 1		Expt 2	
		Avg migration[c]	MMI ± SD[d]	Avg migration[c]	MMI ± SD[d]
Ts 1 survivors	Control (medium)	77.5	100 ± 4	77.5	100 ± 4
	$CVB3_m$-inoculated mice (200)	74.6	96 ± 5	78.7	101 ± 3
	$CVB3_m$-inoculated mice (200)	75.8	98 ± 6	77.2	100 ± 5
	Normal mice (400)	74.3	96 ± 5	79.0	102 ± 3
	$CVB3_m$ lysate [10^8]	74.0	95 ± 5	75.7	98 ± 5
Ts 1 survivors inoculated with $CVB3_m$	Control (medium)	62.2	100 ± 4	85.6	100 ± 5
	$CVB3_m$-inoculated mice (200)	65.6	105 ± 5	86.2	101 ± 5
	$CVB3_m$-inoculated mice (400)	61.2	98 ± 5	86.5	101 ± 5
	Normal mice (400)	64.3	103 ± 5	86.2	101 ± 5
	$CVB3_m$ lysate [10^8]	56.2	90 ± 5	85.6	100 ± 5
Normal mice	Control (medium)	85.9	100 ± 6	ND[e]	
	$CVB3_m$-inoculated mice (200)	85.6	99 ± 5	ND	
	Normal mice (200)	87.2	102 ± 4	ND	
	Normal mice (400)	86.6	101 ± 5	ND	
	$CVB3_m$ lysate [10^8]	86.3	101 ± 5	ND	
Normal mice inoculated with $CVB3_m$	$CVB3_m$-inoculated mice (200)	53.1	57 ± 4	45.3	61 ± 5
	$CVB3_m$-inoculated mice (400)	41.6	45 ± 4	34.4	46 ± 5
	Normal mice (400)	93.4	100 ± 4	76.2	102 ± 5
	$CVB3_m$ lysate [10^8]	32.2	35 ± 4	25.9	35 ± 5

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

[b] Antigens tested per 0.1 ml were KCl extracts of heart tissues from CD-1 mice inoculated i.p. with 10^7 PFU of each virus, or heart tissues from normal mice, or a HeLa cell virus lysate which contained approximately 160 μg per 0.1 ml and 10^8 PFU.

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

[d] MMI = [(Distance of cell migration with challenge antigen)/(Distance of cell migration without challenge antigen)] × 100 ± standard deviation. The error was calculated according to the method of Paque et al. (33). An MMI of ≤65% is considered a statistically significant value (2).

[e] ND, Not determined.

determined to be T lymphocytes by the following experiments. Splenic lymphoid cells from several populations were treated with anti-theta antiserum and complement, as described above. Data on cells from four individual mice (no. 3, no. 7, etc.) which exhibited a positive reaction (SI >2.0) to purified $CVB3_m$ particles (original data are summarized in Table 6) are presented in Table 7. In all four cell populations, removal by lysis of T lymphocytes with anti-T lymphocyte serum and complement abrogated the response of the cell populations to $CVB3_m$ particles. These data suggested that the [^{3}H]thymidine incorporation was due to a T-lymphocyte response. To further exclude B-lymphocyte responses in these assays, we performed the following experiment (data not shown). A portion of each of the four cell populations shown in Table 7 was treated with mitomycin C (50 μg/ml for 45 min at 37°C) followed by three washes in complete medium. A sample of these cells was mixed with cells from the same population which were depleted of T cells by treatment with anti-T cell serum and complement (1:2 ratio of cell numbers, respectively). This mixture of cells (B cells and nondividing T and B cells) from each of the four cell populations was plated at approximately 2.5×10^5 cells per well in the absence or presence of purified $CVB3_m$ particles, and the [^{3}H]thymidine incorporation assay was performed. The results showed that these mixed-cell cultures were also unresponsive (SI of 0.2 to 1.6, with incorporation at 12 to 120 cpm in response to purified $CVB3_m$ particles). Also, treatment of the four cell populations with anti-T cell serum and complement abrogated the SI response (SI of 0.1 to 1.6) to purified virus particles. Incubation of the lymphocytes with only anti-T lymphocyte serum or only complement did not have any effect on the response of the lymphocytes to purified virus particles in the [^{3}H]thymidine incorporation assay (data not shown). Since samples of these four untreated cell populations responded to $CVB3_m$ particle antigens but failed to respond when treated or mixed as described in either situation described

TABLE 6. Stimulation indices of splenic lymphoid cells from control or $CVB3_m$-challenged normal and *ts* 1 survivor mouse cells incubated with purified $CVB3_m$ virus particles[a]

Mice	Challenge with $CVB3_m$	Response to $CVB3_m$ antigens (range of SI ± SD)[b]	Number of Mice with SI[c,d]	
			<2.0	>2.0
Normal	−	0.896 ± 0.045 to 2.306 ± 0.479	13	1
	+	0.656 ± 0.053 to 2.044 ± 0.254	10	4
Ts 1	−	1.044 ± 0.136 to 8.410 ± 1.136	7	6
	+	0.950 ± 0.067 to 5.198 ± 0.414	7	6

[a] Adolescent mice were challenged with 10^7 PFU i.p. and sacrificed at 8 days p.i. to obtain the splenic lymphoid cells.

[b] SI (stimulation index) = (average counts per minute incorporated in the presence of 10^7 PFU)/(average counts per minute incorporated in the presence of medium only). The most disparate values were selected from several experiments to show the range.

[c] Stimulation indices of 2.0 or more were considered positive.

[d] A chi-square test of all normal mice (with or without $CVB3_m$) versus all *ts* 1 survivors (with or without $CVB3_m$) for the difference between these two groups relative to SI less than or greater than 2.0 was significant ($P < 0.05$).

above, we conclude that splenic T lymphocytes are the cells responding immunospecifically.

Reactivity of cytotoxic T lymphocytes from unchallenged or $CVB3_m$-challenged *ts* 1 survivors against uninfected or $CVB3_m$-infected CD-1 neonatal skin fibroblasts. Previous studies by Huber et al. (21), Wong et al. (51–53), and Woodruff (54, 55) established that adolescent mice challenged with $CVB3_m$ generate cytotoxic T lymphocytes which are reactive against $CVB3_m$-infected target cells. It was thus of interest to determine whether *ts* 1 survivors generated a similar population of immunoreactive cells. We used only male adolescent mice in these studies, as cytotoxic T cells are poorly or not at all generated by female mice challenged with $CVB3_m$ (53). Assays were performed with T lymphocytes from individual mice, since CD-1 mice are semi-inbred. After sacrifice at 7 days p.i., the nonadherent spleen cell populations of mice from the four uninoculated or $CVB3_m$-inoculated normal or *ts* 1 survivor mice groups were incubated with normal or $CVB3_m$-inoculated normal or *ts* 1 survivor mice groups were incubated with normal or $CVB3_m$-challenged neonatal skin fibroblast target cells. The results of one assay conducted with male mice are shown as experiment 1 in Table 8. Individual mice gave a wide range of responses to both uninoculated and $CVB3_m$-inoculated target cells, but in general, cells from $CVB3_m$-inoculated normal or $CVB3_m$-inoculated *ts* 1 survivor mice reacted with $CVB3_m$-infected target cells to a greater extent than they did with uninfected target cells. Responses of lymphocytes from *ts* 1 survivors (not challenged with $CVB3_m$) were

TABLE 7. Effect of treatment of spleen cells with anti-theta antiserum and complement on their response to purified $CVB3_m$[a]

Source of spleen cells	Cpm ± SD[b]					
	Untreated cells			Treated (anti-theta antibody plus complement) cells		
	Medium only	Purified $CVB3_m$	SI[c]	Medium only	Purified $CVB3_m$	SI[c]
Normal + virus (no. 3)	425 ± 86	1,215 ± 145	2.9	97 ± 23	66 ± 17	0.7
Normal + virus (no. 7)	1,150 ± 175	2,845 ± 566	2.5	66 ± 17	105 ± 29	1.6
Ts 1 survivor (no. 3)	485 ± 48	1,148 ± 151	2.4	112 ± 42	11 ± 2	0.1
Ts 1 survivor (no. 4)	977 ± 39	2,007 ± 120	2.1	176 ± 50	100 ± 34	0.6

[a] Approximately 2.5×10^5 cells were mixed with 10^7 PFU of purified $CVB3_m$ in 0.2 ml total for 3 days at 37°C. On day 3, 0.75 μCi of [^{3}H]thymidine (6 mCi/ml) was added, and incubation continued for 24 h.

[b] Cpm values represent a mean count from three to four microcultures ± standard deviation.

[c] SI (stimulation index) = (mean counts per minute in presence of $CVB3_m$)/(mean counts per minute in absence of virus [medium only]). The mean SI for untreated cells was 2.5 ± 0.3; for treated cells it was 0.8 ± 0.6 ($P < 0.01$) by Student's *t* test.

TABLE 8. Cytotoxic activity of splenic lymphoid cells from uninoculated or $CVB3_m$-inoculated normal or *ts* 1 survivor mice against uninfected or $CVB3_m$-infected CD-1 neonatal skin fibroblasts[a]

Expt	Source of lymphocytes	Mouse	Cytotoxicity (% lysis) of target cells ± SD[b]	
			Uninoculated	$CVB3_m$ inoculated
1	Normal mice	1	−6.7 ± 0.3	−2.9 ± 0.2
		2	−6.7 ± 0.9	5.9 ± 0.4
		3	−6.2 ± 0.7	−1.1 ± 0.1
		4	12.6 ± 1.7	5.3 ± 0.4
	$CVB3_m$-inoculated normal mice	1	−11.9 ± 1.2	1.4 ± 0.2
		2	−12.8 ± 2.0	4.3 ± 0.5
		3	9.4 ± 0.9	5.3 ± 0.6
		4	26.6 ± 7.0	10.9 ± 1.7
	Ts 1 survivors	1	31.2 ± 3.9	34.5 ± 9.9
		2	43.6 ± 4.1	42.7 ± 9.1
		3	51.4 ± 5.8	40.9 ± 13.2
		4	55.7 ± 6.2	69.4 ± 34.5
	Ts 1 survivors inoculated with $CVB3_m$	1	−5.3 ± 0.2	6.6 ± 2.5
		2	−3.9 ± 0.7	8.4 ± 1.0
		3	−2.5 ± 0.4	5.3 ± 1.5
		4	−5.0 ± 0.5	−1.4 ± 0.4
2[c]	$CVB3_m$-inoculated normal mice (treated with anti-theta antibody plus complement)	a	−13.8 ± 2.4 (−16.4 ± 2.2)	10.3 ± 0.9 (0.9 ± 0.1)
		b	−18.4 ± 5.1 (−12.0 ± 1.5)	20.3 ± 1.4 (4.9 ± 0.5)
		c	−3.6 ± 1.1 (−21.7 ± 3.9)	−2.8 ± 0.3 (3.3 ± 0.9)
	Ts 1 survivors inoculated with $CVB3_m$ (treated with anti-theta antibody plus complement)	a	−18.7 ± 1.3 (−23.0 ± 3.1)	5.1 ± 1.0 (−4.4 ± 0.3)
		b	−13.6 ± 3.4 (−27.4 ± 5.2)	21.7 ± 4.8 (0.0 ± 0.1)
		c	3.6 ± 0.5 (−21.5 ± 2.0)	3.5 ± 0.2 (−1.4 ± 0.1)
3[d]	Normal BALB/c mice		6.8 ± 1.0	13.5 ± 0.8
	$CVB3_m$-inoculated BALB/c mice		7.4 ± 1.3	36.7 ± 2.8

[a] Spleen cells were harvested at 7 days p.i., and nonadherent cells were incubated with ^{51}Cr-labeled target cells at an effector-to-target cell ratio of 100 to 150:1 for 20 h at 37°C. Average myocarditic lesion scores and average lesion numbers for the four groups of mice in experiment 1 in descending order, respectively, were 0 (0), 2+ (10.3, with all hearts having at least three lesions), 0 (0), and 0 (0.4). In experiment 2, the lesion score and number averages were 0 (0) and 4+ (83), respectively.

[b] Cytotoxicity was calculated by: [(average percentage of ^{51}Cr released from test group) − (average percentage of ^{51}Cr released from medium control)]/[(average percentage of ^{51}Cr released by freeze-thaw from test group) − (average percentage of ^{51}Cr released from medium control)].

[c] Portions of each cell population were treated with anti-theta antiserum and complement as described in the text. Values obtained after treatment are in parentheses.

[d] Lymphocytes pooled from four normal and six $CVB3_m$-inoculated mice were used in this experiment.

higher in this experiment than we generally measured; most responses of *ts* 1 survivors were at levels found for lymphocytes from $CVB3_m$-inoculated normal mice or $CVB3_m$-inoculated *ts* 1 survivors. In a second experiment (representative data are presented as experiment 2, Table 8), treatment of cells with anti-theta antiserum and complement abrogated the cytotoxic (lytic) activity present in most cell populations from $CVB3_m$-inoculated normal or $CVB3_m$-challenged *ts* 1 survivor mice against uninfected and $CVB3_m$-infected target cells. We examined anti-theta and complement-sensitive cytotoxic activity of seven sera from the former and three sera from the latter groups of mice, respectively. Removal of T cells by this treatment reduced cytolytic activity of all three treated cell populations from the $CVB3_m$-challenged *ts* 1 survivors for both kinds of targets, whereas two treated cell populations from this latter group of mice exhibited increased activity against only $CVB3_m$-infected cell targets. Thus, our data are in partial agreement with previously published data (51, 54), but the inability of anti-theta serum and complement to reduce activity in several sera from $CVB3_m$-inoculated mice may suggest high natural killer (NK) cell activity in those mice (20). To assess our technique, cytotoxic T-lymphocyte assays were also performed with the $CVB3_m$-inoculated BALB/c mouse model, which has been extensively studied by Huber et al. (21), Wong et al. (51–53), and Woodruff (54, 55). Our results (Table 8, experiment 3) are similar to those published and show that T lymphocytes from spleens of $CVB3_m$-infected mice express the highest activity against

$CVB3_m$-inoculated target cells, although some cytotoxicity was directed against the latter target cells by T lymphocytes obtained from spleens of normal mice.

The proportion of the total number of normal and *ts* 1 survivor mice with splenic cytotoxic T lymphocytes active against normal or $CVB3_m$-infected neonatal skin fibroblasts was summarized from several experiments (Table 9). In general, the data show that T lymphocytes from similar proportions of normal or $CVB3_m$-challenged normal mice recognized (percent lysis >0) uninoculated fibroblasts, whereas a significantly higher proportion of $CVB3_m$-challenged mice recognized $CVB3_m$-inoculated target cells. A high proportion of *ts* 1 survivors recognized both uninoculated and $CVB3_m$-inoculated target cells, and the extent of response to uninoculated and $CVB3_m$-inoculated targets was about the same. Inoculation of normal mice or *ts* 1 survivors with $CVB3_m$ resulted in a greater proportion of mice in either population responding to $CVB3_m$-uninoculated target cells. Statistical analysis of the mean percent lysis values for lymphocytes from all individuals in a group against uninfected or $CVB3_m$-infected target cells revealed that they were unequal. Student's *t* test analyses of means from paired groups showed that splenic lymphocytes from normal mice reacted more strongly against $CVB3_m$-infected targets than against normal targets ($P < 0.026$). However, lymphocytes from $CVB3_m$-inoculated normal mice were heterogeneous in their response, as previously reported (20), and the grand means of reactivity against uninfected or $CVB3_m$-infected targets was not significantly different at the 95% confidence level ($P < 0.07$). Reactivity of *ts* 1 survivor mice lymphocytes was similar to that of both kinds of target cells, whereas challenge of *ts* 1 survivors with $CVB3_m$ resulted in enhanced reactivity against $CVB3_m$-infected targets ($P < 0.002$). It must be noted that in experiments involving 20 $CVB3_m$-inoculated normal mice, we did not find a correlation between the severity of myocarditis induced by $CVB3_m$ and the relative extent of cytotoxic T-cell response measured in vitro.

Production of interferon by *ts* 1 survivors and $CVB3_m$-inoculated *ts* 1 survivors. The fact that *ts* 1 survivors possess T lymphocytes which recognize $CVB3_m$-induced antigens suggested that *ts* 1 survivors produce gamma-type interferon which could contribute to their apparent resistance to $CVB3_m$ induction of myocarditis. The gamma type of interferon is produced by sensitized lymphoid cells upon exposure to the sensitizing antigens (44). It was found in two experiments that splenic lymphoid cells from *ts* 1 survivors, whether inoculated with $CVB3_m$ or not, produced low levels of interferon (40 U) after 1 day of incubation with $CVB3_m$ antigens, but interferon was not detected (<10 U) after 5 days in culture. Splenic leukocytes from $CVB3_m$-inoculated normal mice produced similar levels of interferon (20 to 40 U) after 1 day of incubation with $CVB3_m$ antigens. Lymphoid cells from normal mice did not produce any interferon in response to $CVB3_m$ antigens. These results suggest that $CVB3_m$-induced myocarditis cannot be explained by a differential

TABLE 9. Proportion of uninoculated or $CVB3_m$-inoculated *ts* 1 survivor or normal adolescent mice possessing splenic lymphocytes which exhibit cytotoxicity during incubation with uninoculated or $CVB3_m$-inoculated neonatal CD-1 skin fibroblasts[a]

Source of splenic lymphocytes[b]	Proportion of animals with lymphocytes cytotoxic against:[c–r]					
	Uninfected fibroblasts			$CVB3_m$-infected fibroblasts		
	No. positive/ total	Proportion of animals	Mean % lysis (±SEM)	No. positive/ total	Porportion of animals	Mean % lysis (±SEM)
Normal mice	5/11[c]	0.45	−5.7 ± 4.9[k]	4/11[d]	0.36	4.0 ± 3.5[l]
Normal mice + virus	7/20[e]	0.35	0.6 ± 5.5[m]	17/20[f]	0.85	8.3 ± 3.3[n]
Ts 1 survivors	14/20[g]	0.70	8.1 ± 8.0[o]	9/20[h]	0.45	9.1 ± 6.1[p]
Ts 1 survivors + virus	6/23[i]	0.26	−12.9 ± 4.7[q]	20/23[j]	0.87	7.0 ± 1.4[r]

[a] Animals with lymphocytes exhibiting cytotoxic reactivity (percent lysis >0).

[b] Splenic cells harvested from mice sacrificed at 7 days after inoculation of virus.

[c–r] Proportion of animals with positive lymphocyte reactivity to the total number of animals tested against that particular target cell. Significance was calculated by chi-square test with Yates correction factor: for *e* versus *f*, $P < 0.005$; for *i* versus *j*, $P < 0.001$; for *c* versus *g*, $P < 0.1$; for other comparisons of the proportion of animals in groups with positive cytotoxic lymphocytes. $P < 0.2$. Analysis of variance of the means showed that they were not equal ($P < 0.017$). A two-tailed Student *t* test on the means showed that for *k* versus *l*, $P < 0.026$; for *m* versus *n*, $P < 0.07$; for *o* versus *p*, $P > 0.3$; and for *q* versus *r*, $P < 0.002$.

production of interferon by *ts* 1 survivors compared with normal mice after challenge with $CVB3_m$.

DISCUSSION

Participation of immunological mechanisms in CVB3 induction of murine myocarditis is well established. Specifically, T lymphocytes are thought to be important in development and formation of the myocarditic lesion (see review, reference 55). The absence of myocarditic lesions in $CVB3_m$-challenged *ts* 1 survivors, in contrast to the induction of lesions by $CVB3_m$ in normal mice, suggested an altered immune response to $CVB3_m$ infection in adolescent *ts* 1 survivors. The data presented herein suggest that the resistance to $CVB3_m$-induced myocarditis in *ts* 1 survivors is explained by distinct differences from normal mice in immunological responses relative to: (i) rapid and high-titered production of circulating anti-$CVB3_m$ neutralizing antibody, and (ii) altered (compared with normal mice) T-lymphocyte responses to $CVB3_m$ virion antigens and $CVB3_m$-induced immunoreactive murine heart tissue antigens.

In addition, the more rapid synthesis and higher titers of neutralizing antibody from $CVB3_m$-challenged *ts* 1 survivors compared with normal mice challenged with $CVB3_m$ suggest that these mice are experiencing an anamnestic response to CVB3 antigens. Studies on the class of antibody evoked after $CVB3_m$ challenge of *ts* 1 survivors are in progress. Neonatal mice are born with functional B lymphocytes (29) and have the capacity for production of specific antibody to many antigens at birth or within a few days after birth (1, 7, 8, 35, 46).

Although neutralizing antibody is regarded as an important host defense in preventing disease in mice challenged with picornaviruses (28, 30), inhibition of replication of CVB3 in target organs, including the heart, cannot be attributed solely to neutralizing antibody (55). Passive transfer of anti-$CVB3_m$ neutralizing antibody to suckling mice can prevent death upon subsequent parenteral challenge with CVB3 (37). However, increased vulnerability with age of adult versus adolescent mice to CVB3-induced myocarditis could not be correlated with differences in amounts of CVB3-neutralizing antibody synthesized; in fact, neutralizing antibody titers were slightly higher in susceptible adult mice (41). Studies by Woodruff (54), who utilized cortisone treatment of mice to interfere with release of monocytes and their mobilization to infected target cells, suggested that inhibition of CVB3 spread by neutralizing antibody is secondary to a monocyte-mediated antiviral activity. Results of an earlier study (36), in which both CVB3-neutralizing antibody and PEC from CVB3-immune mice were required to protect suckling mice from death due to CVB3, also suggest the requirement for cell-mediated immunity in protection against CVB3. Our results, in which virus titers in heart tissues of $CVB3_m$-challenged *ts* 1 survivor and normal mice were compared, showed no significant differences. Thus, the replication of virus in heart tissues was not inhibited by the presence of a 10-fold-greater level of CVB3-neutralizing serum antibody in $CVB3_m$-challenged *ts* 1 survivors, indicating that CVB3-neutralizing antibody alone cannot account for the reduction in myocarditis seen in *ts* 1 survivors. In separate experiments, normal mice challenged with $CVB3_m$ and given 20 U of anti-$CVB3_m$ antibody on day 3 p.i. and 200 U of the same antibody on day 6 p.i. exhibited extensive myocarditis by day 8 p.i., similar to that observed in heart tissues of $CVB3_m$-challenged mice (Gauntt, unpublished data). Thus, mimicry of anti-$CVB3_m$ antibody levels in $CVB3_m$-challenged *ts* 1 survivors is insufficient in normal mice to prevent myocarditis.

A role for cell-mediated immunopathology in the pathogenesis of CVB3-induced myocarditis in mice is suggested by two lines of evidence. First, Wong et al. (51, 52), Woodruff (54), and Huber et al. (20, 21) showed that mice which were infected with CVB3 under conditions leading to myocarditis also developed cytotoxic T lymphocytes, detectable in 3 to 4 days, which reacted with both uninfected and infected target cells, but which by day 7 were reactive almost entirely with infected targets. Secondly, studies by Woodruff and Woodruff (56) and by Roesing et al. (40), respectively, showed that when thymectomized or nude (athymic) mice were infected with CVB3, replication of CVB3 occurred in heart tissues and virus was cleared as in normal mice, but without induction of myocarditic lesions. Mice are born with lymphocytes possessing the theta antigen (6, 45), and shortly after birth, these cells can participate in cell-mediated immune reactions (7, 35, 45). Thus, *ts* 1 survivors should possess cell-mediated immune capabilities at the time of first encounter with *ts* 1 virus at birth, although immunocompetency at birth was not examined.

In the present study, T-cell reactivities were measured against CVB3-induced antigens in three different in vitro assays: cytotoxicity, migration inhibition, and stimulation of [^{3}H]thymidine uptake. We readily measured cytotoxic T-cell responses against both uninoculated and $CVB3_m$-inoculated neonatal skin fibroblasts with splenic lymphocytes taken from adolescent *ts* 1 survivors. The cytotoxic response of lymphocytes from the majority of *ts* 1 survivor mice appeared to shift from being reactive against

both uninfected and infected target cells to reacting more specifically against infected fibroblasts after in vivo challenge with $CVB3_m$. Thus, after infection with $CVB3_m$, *ts* 1 survivors exhibited cytotoxic T-cell responses very similar to those of $CVB3_m$-challenged normal mice.

On the other hand, our results from the agarose droplet cell migration inhibition assay showed that PEC from *ts* 1 survivors did not appear to react with either virus particles or $CVB3_m$-induced antigens from murine heart tissues, whereas PEC from normal mice inoculated with $CVB3_m$ reacted with both antigen preparations (see also 33, 34, 48). This suggests that either the PEC from *ts* 1 mice were unresponsive to $CVB3_m$ virion and virus-induced cellular antigens, or that they responded in a manner other than that required to produce migration inhibitory factor (MIF), the lymphokine measurable in this assay. The failure of PEC from *ts* 1 mice to demonstrate MIF reactivity in response to CVB3 particle or CVB3-induced antigens may indicate that (i) the T-cell recognition of both virion or altered cell antigens and major histocompatibility complex products is required (58) and (ii) one or both sets of the two groups of antigens are altered in *ts* 1 survivors. Alternatively, altered macrophage-monocyte reactivity may account for the lack of delayed hypersensitivity which parallels the lack of $CVB3_m$-induced heart lesion seen in $CVB3_m$-challenged *ts* 1 mice compared with $CVB3_m$-challenged normal mice. Our results also imply a difference in the way in which the anti-CVB3 immune response is regulated, i.e., $CVB3_m$-inoculated *ts* 1 survivors produced neutralizing antibody to high titer, but effector cells in their PEC population did not release MIF; in contrast, $CVB3_m$-inoculated normal mice produced little neutralizing antibody, but effector cells in their PEC population were able to release MIF in response to antigenic stimulation. Such altered reactivity could be regarded as a form of immune deviation in which the humoral immune response is preferentially stimulated. Immune deviation of this type has been shown in other systems to be due to the activation of suppressor cells (10). Consistent with the concept of suppressor cell-mediated immune deviation was the finding that T cells from the spleens of *ts* 1 survivors reacted strongly in stimulation assays against purified $CVB3_m$ particle antigens, whereas splenic T cells from normal mice did not exhibit reactivity. It has been demonstrable in the human system, by employing cell-mixing experiments, to show that blastogenesis of T cells in the absence of cytotoxic reactivity is associated with suppressor cell activation (38).

Cell-mixing experiments between lymphocytes from normal mice and *ts* 1 survivors to measure suppressor cell activity have not been performed because our model has been established with the CD-1 semi-inbred mouse line. Other strategies that may be used to assess possible suppressor cell differences in the two groups of mice include (i) enumeration of cells bearing surface markers characteristic of the suppressor population, such as Lyt 2^+3^+, Ia-K, and IgG receptors (31) and (ii) eliminating the T suppressor cell population in *ts* 1 survivors by low-level cyclophosphamide treatment (41) before challenge with $CVB3_m$. In preliminary studies (Gauntt, unpublished data), it was found that treatment of *ts* 1 survivors with 25, 50, 150, or 250 mg/kg of cyclophosphamide 4 h before inoculation of $CVB3_m$ resulted in easily demonstrable myocarditis in the majority of animals; *ts* 1 survivors challenged with virus alone had no evidence of myocarditis. Thus, these results suggest that *ts* 1 survivors possess suppressor cell activity that may be contributing to resistance. In other studies, the proportions of lymphocytes bearing Lyt 1 and Lyt 2 surface antigens were compared by using fluorescent monoclonal anti-Lyt 1.1, 1.2, 2.1, and 2.2 antibody, and the results showed no difference in proportions between *ts* 1 survivors and normal mice (data not shown). These latter data parallel and are in agreement with other data showing that infection of syngeneic susceptible C57BL/6 mice with $CVB3_m$ does not result in marked differences in proportions of T-lymphocyte subsets in the thymus, spleen, peripheral blood, peritoneal cavity, or lymph node populations between 3 and 12 days p.i. (R. E. Paque, C. J. Gauntt, and M. McKown, 1982. Abs. Fed. Am. Soc. Exp. Biol. p. 565 Abs. no. 1727. 66th Annual Meeting, New Orleans, La.). We are presently experimenting with several inbred mouse lines to establish a suitable inbred mouse model that would permit use of adoptive transfer assays in vivo and cell mixing experiments in vitro to answer this question.

At present, four distinct cell-mediated mechanisms capable of killing virus-infected target cells are known (43). Of these, only direct T-cell-mediated cytotoxicity has been studied in the CVB3-myocarditis model in mice, because this mechanism is thought to be involved in the production of myocarditis. Both *ts* 1 survivors and normal mice challenged with $CVB3_m$ had similar levels of T-cell cytotoxicity against CVB3 antigens and against normal tissue antigens. Thus, it is unlikely that cytotoxic T cells alone account for the amyocardic status of *ts* 1 survivors. Other cell-mediated mechanisms that may be important include antibody-dependent cell cytotoxicity, NK cell-mediated cytotoxicity, and macrophage-mediated cytotoxicity, none of which has been directly tested in the

CVB3-murine myocarditis model. Data from the present study suggest that similar levels of interferon are produced in *ts* 1 and normal mice after challenge with $CVB3_m$. Because NK cells are activated by interferon (9, 18, 19, 57), it seems unlikely that a differential activation of NK cells could account for the difference in myocarditis, because splenic leukocytes from *ts* 1 and normal mice produced similarly low levels of interferon in response to $CVB3_m$ antigens in vitro. However, in view of our present findings, which suggest a difference in T-cell activation in *ts* 1 versus normal mice after challenge with $CVB3_m$, coupled with the failure of *ts* 1 spleen cells to release MIF when challenged with $CVB3_m$ and the indirect data of Woodruff (55) suggesting that monocytes were important in the immune response to CVB3, we consider that activated cytotoxic macrophages, which are far less specific than other cytotoxic effector cells, may be responsible for the induction of myocarditic lesions in susceptible $CVB3_m$-infected mice through an "innocent bystander" mechanism. Data from our present study are consistent with the idea that resistant *ts* 1 survivor mice are protected by an immune deviation mechanism in which T cells may be inhibited in release of macrophage activation lymphokines, resulting in reduced macrophage-mediated tissue damage. Proof of these mechanisms requires demonstration of macrophage-mediated cytotoxicity against CVB3-infected and normal murine targets as well as identification of the mechanism by which macrophages are activated or blocked from activation in this model. It will also be necessary to assess the contribution of other cytotoxic mechanisms that may be operative, such as NK, antibody-dependent cell-mediated cytotoxicity, or the contribution of a newly described murine splenic cell type which is an accessory cell required for cytolytic T-lymphocyte response to viral antigens in vitro (12). This latter unidentified cell is glass and nylon wood adherent and radiosensitive, and it lacks surface immunoglobulin and Thy 1.2 antigen (12). Experiments have been initiated to examine these questions.

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