Specific and Nonspecific Heart Defenses in Enteroviral Infections

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Introduction

Enterovirus infections of humans are common [31], with the majority being asymptomatic [6]. These viruses, particularly the coxsackieviruses group B (CVB) are associated with diseases of the heart [26, 27], with acute myocarditis occurring in up to 5% of infected individuals [26, 40]. Neonatal myocarditis is a severe disease and contributes to mortality in infants and young children [6, 26, 27]. Among the CVB, coxsackievirus B3 (CVB3) is most frequently associated with acute myocarditis [34]. Acute disease can progress to chronic myocarditis, and clinical laboratory data suggest that some cases of chronic disease develop into idiopathic dilated cardiomyopathy [26, 27, 33, 35].

The ubiquity of the CVB among humans is well documented [21, 30]; neutralizing antibodies to several of five CVB serotypes are found in most individuals [21, 31]. Homotypic neutralizing IgM or IgG antibodies are present in sera of humans infected with a single enterovirus; heterotypic IgM antibodies to several enteroviruses are detected in many of these sera by ELISA [9, 32]. Heterotypic anti-CVB antibodies are not neutralizing and recognize a CVB group-reactive epitope on capsid polypeptide VP1 [25, 32]. Not all antiviral antibodies are of benefit to the host. A subpopulation of viral antibodies may have pathologic consequences for the host, as recent studies of CVB-mouse models of infection/disease suggest that CVB infections induce antibodies which cross-react with normal cells [14, 15, 26, 34, 40]. Infections of humans [3] or mice [2] by a single enterovirus also results in sensitization of T lymphocytes which proliferate in vitro in response to homologous/heterologous enteroviral antigens, suggesting T cell recognition of a common enteroviral antigen [3, 4].

The induction, development, and molecular mechanisms of coxsackievirus-induced acute and chronic myocarditis have been studied in a number of CVB3-mouse models of disease [7, 24, 26, 27]. These excellent

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models exhibit many parallel events which occur in humans. Age of a mouse experiencing a CVB3 infection is a major determinant of disease: younger mice die from infections by temperature-sensitive mutants, amyocarditic variants, or myocarditic variants of CVB3 [10, 37]. In young mice, immunogenetic background determines rate of production of protective antibodies, a response which significantly affects the extent of cardiopathology induced [23, 39]. Genetic background of a mouse can also predict death from a CVB3 infection; several inbred strains die within a week of challenge [7, 26]. Adolescent/adult mice of inbred strains develop acute myocarditis due to a myocarditic CVB3 variant, but extent of cardiopathology depends upon determinants located outside of the 5′ terminus nontranslated region [37] and genetic background of the murine strain [7, 10, 24, 26]. Murine strains which succumb to development of CVB3-induced chronic myocarditis possess certain major histocompatibility complex (MHC) and non-MHC determinants which are currently being defined [5, 27]. Thus age and genetic background are major host factors predisposing mice to chronic myocarditis due to infection by a myocarditic CVB3 variant.

During initial infection of mice with a myocarditic CVB3 variant, several nonspecific host defense mechanisms respond within 2–4 days postinoculation (p.i.), including synthesis of arachidonic acid cascade products [13, 16], lymphokines and cytokines [6], interferon-β and -γ [12, 18], activation of natural killer (NK) cells [18], and adsorption of virus by leukocytes [12]. In acute myocarditis, arachidonic acid cascade products [13, 16] and interferon-γ [12] continue to be produced, and a small number of NK cells are activated. These nonspecific responses have not been described in tissues during chronic disease.

The cell-mediated immune system responds to a CVB3 infection by production of T lymphocytes specific for antigens on cultures of virus-infected and uninfected cells [24, 26, 38]. In vitro, cytotoxic T lymphocytes lyse virus-infected and uninfected targets and proliferate in response to a group-specific enterovirus antigen(s) [1–4] found on several different enterovirus particles [1]. T lymphocytes are found in myocarditic heart tissues of infected mice [19]. It is not known which subset(s) of T lymphocytes [24, 38] is present in heart tissues of mice with CVB3-induced chronic myocarditis [5, 23, 34, 40].

The humoral immune system responds rapidly to a CVB3 infection in mice: protective antiviral antibodies can be detected by day 3 p.i. [5, 26, 39], and titers remain high during acute and chronic diseases [5, 40]. Some murine strains with CVB3-induced chronic myocarditis produce antibodies which bind antigens on normal heart tissues [34, 39, 40]. A portion of these antibodies recognize epitopes on cardiac myosin, an immunogen which can induce an acute myocarditis in certain inbred strains [30, 34], data suggesting potential cross-reacting epitopes on heart tissues. Hyperimmune anti-CVB3 antisera contains antibodies capable of exacerbating CVB3-induced acute myocarditis [17]. Studies of anti-CVB3 neutralizing monoclonal antibodies (mAbs) show that some mAbs can participate in potential pro-inflammatory reactions in vitro, and other mAbs can induce cardiopathologic

alterations in vivo [11, 14]. Thus, published data show that autoimmune responses, with cardiopathologic consequences, can be attributed to both cell-mediated and humoral immune systems. These data imply that immune systems recognition of viral epitopes shared with heart tissues can lead to autoimmune (chronic) myocarditis in mice, and perhaps in humans, of specific genetic backgrounds.

Materials and Methods

$CVB3_m$, a highly myocarditic variant of CVB3, was propagated and assayed in HeLa cells as previously described [18], as well as other CVB serotypes whose origins have been described [20]. ^{35}S-labeled coxsackievirus B2 (CVB2) was prepared as follows. HeLa cells were incubated with 50–100 plaque-forming units (PFU) of virus/cell for 1 h at 37°C. MEM containing 1% fetal bovine sera was added and the cells incubated for an additional 2 h at 37°C. The culture fluids were replaced by methionine-free MEM with 1% fetal bovine serum for 45 min. Trans-^{35}S-label (ICN Biomedicals, Irvine, CA; Sp. Act. 1085 Ci/mmol) was added at 100 μCi/ml and the cells incubated for 15–18 h at 37°C in a humidified CO_2 incubator. Culture fluids and cell debris were processed for virus particles, including banding of particles in CsCl gradients, as previously described [18]. Purified ^{35}S-labeled CVB2 particles were dialyzed versus Dulbecco's phosphate-buffered saline (DPBS), pH7.4, and stored at −20°C. Specific infectivity of several preparations varied from 500 to 2253 PFU/cpm. Experiments on rate zonal centrifugation of ^{35}S-labeled CVB2 particles ± an anti-$CVB3_m$ neutralizing mAb were performed as follows. mAb-virus particle mixtures were layered over preformed 10%–40% sucrose gradients (sucrose was dissolved in DPBS) and centrifuged for 2.5 h at 32K rpm in a Beckman SW50.1 rotor at 5°C. Fractions were collected from the bottom and assayed for infectivity by a plaque method, cpm by a β spectrometer, and percentage sucrose by an Abbe 3L refractometer (Bausch and Lomb).

Hybridomas producing neutralizing anti-$CVB3_m$ mAbs were previously described [11] and were propagated in Dulbecco's MEM containing 10% fetal bovine serum and antibiotics. All mAb preparations were assayed for anti-$CVB3_m$ neutralizing antibody content by a cytopathic effects (CPE) inhibition assay [11], and those with titers of at least 8 were used. Isotyping was performed with commercial kits. mAbs were purified by affinity chromatography using affinity-purified goat anti-mouse IgG bound to agarose beads (Sigma Chemical, St. Louis, MO). IgG concentrations were determined by a dye-binding method (BioRad Laboratories, Richmond, CA). Protein A-purified mAb HB79 (anti-DNP) was a gift from Judy Teale, University of Texas Health Science Center.

Adolescent (4–6 weeks old) CD-1 or C3H/HeJ mice were purchased from Charles River Breeding Laboratories (Boston, MA) or Jackson Laboratories (Bar Harbour, ME), respectively. Mice were given water and mouse chow ad libitum. Mice were inoculated with virus by intraperitoneal

route. Sacrifice of deeply anesthesized (ether) mice was by cervical dislocation. Hearts were quickly removed and fixed in 10% buffered formalin, and coronal sections were stained with hematoxylin/eosin. Sections were examined for focal lesions containing necrotic myocytes and inflammatory leukocytes at 40 and 100 [18].

Results

Virus-neutralizing antibodies in serum are a major factor in terminating primary infections/diseases and in providing protection against subsequent illnesses/diseases induced by the same virus serotype through reducing spread of virus within the host. Our findings with two anti-CVB3 neutralizing mAbs show that such mAbs can participate in other reactions which are not in the best interests of the host.

Properties of Anti-CVB3$_m$ Neutralizing mAbs 14 and 24

Both mAbs are of the IgG2a subclass. mAb 24 but not mAb 14 participates in complement-mediated lysis of normal mouse fibroblasts [11, 14, 15]. Both mAbs induce production of a soluble chemoattractant for unelicited macrophages from normal mouse fibroblasts [11]. Intraperitoneal inoculations (nine inoculations every other day with $\approx 1\,\mu g$ mAb) of either mAb into normal adolescent male C3H/HeJ mice can result in pathologic alterations in heart tissues [11, 14]. mAb 24 but not mAb 14 binds to rabbit skeletal muscle myosin [11].

Binding of mAbs 14 and 24 to CVB2 Particles

Virus-neutralization assays performed with these mAbs and all six CVB serotypes confirmed neutralization of only CVB3 [11]. However, in these CPE inhibition assays it was observed that HeLa cells incubated with CVB2-mAb 14 or CVB2-mAb 24 complexes exhibited CPE many hours in advance of cultures challenged with only CVB2. These reproducible observations led to the following experiments.

Incubating CVB2 virions with these mAbs prior to challenge of HeLa cells enhanced yields in comparison to cells challenged with CVB2 alone (Table 1). In the initial experiment, either mAb enhanced yields 18- to 65-fold. In experiment 2, different concentrations of purified mAbs were adsorbed to CVB2 prior to challenge of cells. At $125\,\mu g$/ml mAb, yields were at least 100-fold above that of the virus control and 12.5 to $3.1\,\mu g$/ml mAb generally enhanced yields by approximately ten-fold above the virus control. HeLa cells incubated with either mAb alone prior to challenge with CVB2 gave virus yields similar to the virus control. These data suggest that mAb-induced enhancement of virus yields was due to increased adsorption of CVB2 virions.

Mechanisms by which these mAbs might enhance yields of CVB2 from

Table 1. Effect of anti-CVB3$_m$ neutralizing mAbs on CVB2 yields in HeLa cells: virus yield as fold-increase above virus control at mAb concentrations (μg/ml)

Experiment 1				(1–5)		
mAb 14				65		
mAb 24				18		
Experiment 2	(25)	(12.5)	(6.2)	(3.1)	(1.5)	(0.75)
mAb 14	125	9	14	20	2	0.3
mAb 24	240	19	14	7	4	3

mAb- or DMEM-CVB2 (0.4–1 × 10^5 PFU) mixtures were incubated at 37°C for 45 min and added to HeLa cell monolayers at 1 PFU/25–50 cells. After 1 h at 37°C the monolayers were washed three times and incubated in MEM (1% fetal bovine serum + antibiotics) for 24 h (experiment 1) or 8 h (experiment 2). Cells were frozen, thawed three times, and assayed for virus by the plaque method. Virus titers in control cultures were 5.5 × 10^5 (experiment 1) or 2.4 × 10^2 (experiment 2) PFU/ml.

Table 2. Effect of mAb 24 on binding of CVB2 to HeLa cell cultures

		Percentage cpm in virus bound to HeLa cells after incubation with:		
Experiment no.	Adsorption conditions	mAb24	mAb HB79	Medium
1	4°C, 2 h	1.0 ± 0.1	2.7 ± 0.1	3.0 ± 0.1
2	37°C, 15 min	3.9 ± 0.3	2.0 ± 0.2	2.4 ± 0.1
3	37°C, 1 h	13.8 ± 2.5[a]	5.8 ± 1.8	4.4 ± 0.4

Antibodies, mAb 14 (IgG2a, 1–5 μg/ml) or mAb HB79 (anti-DNP, IgG2a, 2 μg/ml), or hybridoma culture medium were incubated with ≈100 000 cpm of CsCl-banded ^{35}S-labeled CVB2 at various times and temperatures, with shaking. Triplicate monolayer cultures of HeLa cells (2.5 × 10^5/well) were challenged with ≈20 000 cpm in 0.2 ml under the specified conditions above. The inocula were removed and the cultures washed three times with medium, collected, and counted in a β spectrometer.

[a] Significantly different from either percentage value obtained with mAb HB79 or medium (Student's t test, $p > 0.05$).

HeLa cells were examined as follows. Data on mAb 24 increasing adsorption of ^{35}S-labeled CVB2 to monolayer cultures of HeLa cells are presented in Table 2. At 4°C, very little CVB2 was bound, whether mAb was present or not. At 37°C, CVB2 adsorption was enhanced by mAb 24 during 1 h of incubation, providing one explanation for the more rapid CPE observed in HeLa cells challenged with CVB2-mAb complexes compared with cells challenged with CVB2 alone.

Rate zonal ultracentrifugation analyses of mAb-purified ^{35}S-labeled CVB2 particle complexes in dense buffered sucrose gradients suggested a second mechanism to explain mAb-increased virus yields. During ultracentrifugation in sucrose gradients, purified CVB2 particles were detected by ^{35}S cpm and infectivity in two populations; one population sedimented as a band at approximately 35% sucrose and the second population at approximately 26% sucrose. Studies of many picornavirus particles separated

Table 3. Rate zonal sedimentation analysis of ^{35}S-labeled CVB2 particle-anti-CVB3 neutralizing mAb complexes in sucrose density gradients

CVB2 particles incubated with:	CVB2 particles sedimenting at ≈35% sucrose			CVB2 particles sedimenting at ≈26% sucrose		
	Total infectivity recovered ($\times 10^6$)	Total cpm recovered	Specific infectivity (PFU/cpm)	Total infectivity recovered ($\times 10^5$)	Total cpm recovered	Specific infectivity (PFU/cpm)
mAb14	189	28659	6595	176	5038	3493
mAb24	92	25805	3565	95	4406	2156
mAb HB79	41	11873	3453	39	10970	356
(anti-DNP)	76	11617	6542	26	10329	252
medium						

CsCl-banded ^{35}S-labeled CVB2 particles were incubated with 1–5 μg mAb 14 or mAb 24 or 2 μg mAb HB79 in hybridoma culture medium or hybridoma culture medium alone for 1 h at 37°C with shaking prior to centrifugation into dense sucrose gradients, as described in "Materials and Methods."

Table 4. Radioimmunoprecipitation of ^{35}S-labeled CVB2 by anti-CVB3$_m$ neutralizing mAbs

^{35}S-labeled CVB2 particles reacted with:	cpm ppt.[a] (% total)	cpm ppt. by mAb/cpm ppt. by antisera E
mAb 14	28.5	55.0
mAb 24	12.3	23.8
Antisera E	51.7	100.0
Medium	3.9	7.5

CsCl-banded ^{35}S-labeled CVB2 particles + mAb (1–5 μg/ml) or antisera E hyperimmune murine anti-CVB3$_m$ (CsCl-banded virus) antisera (1:100 dilution), were incubated for 2 h at 25°C; mixtures were then incubated with affinity-purified goat anti-mouse IgG coupled to agarose for 4 h at 25°C. All precipitates were washed five times with Dulbecco's PBS, and cpm were determined in a β spectrometer. Specific infectivity of ^{35}S-labeled CVB2 was 10.4 PFU/cpm.

[a] Total cpm/sample = 30968.

by this methodology have shown that slower sedimenting virus particles contain mostly empty capsids (no RNA) aggregated with infectious particles, whereas faster sedimenting virus particles are mostly infectious with few empty capsids. The data (Table 3) show that particles (± bound mAb) sedimenting at ca. 35% sucrose in all four gradients had similar specific infectivities (3453–6596 PFU/cpm). CVB2 particles bound to a mAb which sedimented at 26% sucrose had specific infectivities about half of those measured for respective virus particle populations sedimenting at ca. 35% sucrose. In contrast, CVB2 particles incubated with mAb HB79 or medium alone which sedimented at 26% sucrose had specific infectivities 5%–10% of those measured for respective virus populations sedimenting at 35% in these gradients. These data suggest that binding of these two mAbs to

CVB2 particles prevents particle aggregation, thereby increasing infectivity because an aggregate of infectious particles assay as one plaque.

Direct binding of mAb 14 or mAb 24 to CVB2 was demonstrated by immunoprecipitation of purified ^{35}S-labeled CVB2 particles using a mAb and goat anti-mouse IgG coupled to agarose beads (Table 4). Hyperimmune mouse antisera to purified $CVB3_m$ particles (antisera E) and hybridoma medium were included as a positive and negative controls. Using cpm in ^{35}S-labeled CVB2 particles precipitated by polyvalent antisera E as maximum amount bound, mAb 14 bound about one-half and mAb 24 about one-fourth the number of cpm as antisera E, levels three- to sevenfold above background (medium control).

These data suggest a second mechanism by which neutralizing antibodies against one CVB serotype can promote infections/diseases: enhancing infectivity of a different CVB serotype. We are not aware of any published data on this mechanism promoting enterovirus infections/diseases, but it may be operative in the following situation.

Challenge of C3H Mice with CVB2 Particles prior to Infection with CVB3 Exacerbates CVB3-Induced Myocarditis

In an elegant series of experiments, Beck and colleagues [1] showed that sequential CVB infections in young mice can have serious consequences

Table 5. Histopathology in heart tissues of C3H/HeJ mice challenged with CVB2 and subsequently CVB3 (from [1])

Challenge inocula (PFU/mouse)		Mean lesion no./section (day 10)[a]	Comments on histopathology[b]
Primary	Secondary		
None	CVB2 (10^3 or 10^5)	≈3	Small lesions, no calcific deposits, few inflammatory cells/lesion
None	$CVB3_m$ (10^5)	≈10	Small to medium lesions, no calcific deposits, 10–50 inflammatory cells/lesion
CVB2 (10^3 or 10^5)	$CVB3_m$ (10^5)	≈10–25	Small to medium lesions, calcific deposits in many lesions; 50–100 inflammatory cells/some lesions
CVB2 (UV-10^5)	$CVB3_m$ (10^5)	>25	Medium lesions, calcific deposits many lesions; ≥50–100 inflammatory cells/lesion

Male 26-day-old mice were challenged with CVB2; 10 days later some groups were challenged with $CVB3_m$. All mice were 64 days of age at sacrifice.

[a] Day 10 after primary challenge alone or day 10 after secondary challenge (20 days total after primary and secondary challenge).

[b] Lesion size: small, 15–75 μm diameter; medium, 76–200 μm diameter.

above those found with a single infection. Their data [1] (Table 5) show that young male C3H/HeJ mice develop minimal or no myocarditis when challenged with CVB2, but challenge with $CVB3_m$ induces moderate myocarditis. However, a 10-day exposure of mice to CVB2, either infectious or UV-irradiated virus, prior to challenge with $CVB3_m$ significantly exacerbated pathologic alterations in heart tissues. Prior sensitization of animals with CVB2 increased both number of $CVB3_m$-induced myocarditic lesions and lesion size. Exposure of mice to CVB2 prior to $CVB3_m$ also resulted in Ca^{2+} deposition in many lesions, a pathologic feature not found in myocarditic lesions of mice challenged with only $CVB3_m$. Interestingly, while mice inoculated with CVB2 developed significant neutralizing antibody titers to CVB2, challenge of these mice with $CVB3_m$ resulted in a rapid but transient (several days) loss in titer of anti-CVB2 neutralizing antibodies [1]. This finding suggests that anti-CVB2 antibodies may have bound to $CVB3_m$ particles in the challenge inoculum upon entering the bloodstream, possibly contributing to exacerbation of myocardial disease. In contrast, the following data show that mice inoculated with CVB4 before challenge with $CVB3_m$ show some protection against myocardial disease.

Table 6. Effect of sequential CVB4/$CVB3_m$ inoculations upon induction of myocarditis in CD-1 mice

Virus challenge	Day of sacrifice (day p.i.)	Myocarditic lesion number (mean ± SEM)
Experiment 1[a]		
CVB4	7	3.4 ± 1.1
CVB4	14	0.4 ± 0.2
$CVB3_m$	7	35.6 ± 14.1(A)
$CVB3_m$	14	30.3 ± 11.9(B)
$CVB3_m$/CVB4	7	8.1 ± 4.4
CVB4/$CVB3_m$	14	3.3 ± 2.2(A,B)
Experiment 2[b]		
CVB4	14	0
$CVB3_m$	7	11.5 ± 3.9(C)
CVB4/$CVB3_m$	14	0(C)
Experiment 3[c]		
CVB4	7	0
CVB4	14	0
$CVB3_m$	7	31.9 ± 8.4(D)
$CVB3_m$	14	6.1 ± 1.5(E)
$CVB3_m$/CVB4	14	17.9 ± 3.6
CVB4/$CVB3_m$	14	0(D,E)

A,B,C,D, Lesion numbers between sets were significantly different at $p < 0.05$ (Student's t test).

[a] Four female mice/group were inoculated by intraperitoneal route with 5×10^6 PFU of primary, then secondary virus on days 0 and 7, respectively.

[b] Six to seven male mice/group were inoculated by intraperitoneal route with 10^7 PFU of primary, then secondary virus on days 0 and 7, respectively.

[c] Six to eight male mice/group were inoculated by intraperitoneal route with 10^6 PFU of primary or secondary virus on days 0 and 7 respectively.

Adolescent CD-1 Mice Exposed to CVB4 Prior to $CVB3_m$ Have Decreased Myocardial Disease

A CVB4 variant with minimal cardiopathogenicity was used as the primary infecting virus (Table 6). At 7 days p.i. of CVB4, CD-1 mice were challenged with $CVB3_m$, and sacrificed 7 days later. Hearts from mice challenged with CVB4 prior to challenge with $CVB3_m$ consistently (three experiments) showed a reduction in lesion number compared to hearts from siblings challenged with only $CVB3_m$. Heart tissue lesions in CVB4/$CVB3_m$-inoculated mice were considerably smaller in size than myocardial lesions induced with $CVB3_m$ (data not shown). These results were surprising in view of the CVB2/$CVB3_m$ data and show that it is premature to draw general conclusions about heart disease induced during sequential infections with two CVB serotypes. The genetic background of the host likely influenced these different results. C3H mice can progress through $CVB3_m$-induced acute myocarditis into chronic myocarditis, whereas CD-1 mice with $CVB3_m$-induced acute myocarditis undergo healing fibrosis.

Discussion

Heterotypic antibody responses to a CVB serotype other than the infecting serotype are common in humans [9, 32]. The molecular basis for this heterologous anamnestic antibody response is not understood, but similar types of heterotypic antibody responses frequently occur in humans experiencing influenza viruses serotype A infections [28]. Our finding that anti-CVB3 neutralizing mAbs could bind to CVB2 particles and promote higher virus yields from HeLa cells suggests a potential pathologic role for these antibodies. Adsorption of CVB2 to the common CVB receptor on HeLa cells has never been demonstrated, and altering several culture conditions which enhanced binding of CVB3 to HeLa cells did not increase binding of CVB2 particles [8]. CVB2 yields from HeLa cells are low, likely reflecting minimal adsorption [8]. Recently, HeLa cells have been shown to possess an F_c receptor for IgG ($F_{C\gamma}$RIII, 36) which may account for binding and uptake of CVB2 complexes. Enhanced infectivity of dengue virus-heterotypic IgG complexes in vivo occurs by this mechanism, resulting in dengue hemorrhagic fever/shock syndrome [22]. Antibody-dependent enhancement of virus infectivity occurs in flaviviruses, alphaviruses, reoviruses, rhabdoviruses, arenanaviruses, coronoviruses, and retroviruses but has not been reported in picornaviruses [29]. This report is the first to show that neutralizing antibody against one enterovirus can enhance infectivity of a different enterovirus, possibly through binding of virus-antibody complexes to F_c receptors and preventing aggregation of particles.

Although multiple infections of humans by different CVB serotypes occur, the influence of a prior CVB infection on subsequent illness/disease induced by a second CVB has been examined in only a single published

report [1] and experiments reported herein. Exacerbation of disease by heterologous antibody enhancement of infectivity could explain increased myocarditis in CVB2/CVB3-challenged mice. Increased lesion size in these mice could reflect rapid clonal expansion of anti-CVB T cells sensitized to a common enterovirus group antigen during initial infection [2, 4] and their subsequent infiltration during secondary infection, resulting in an increased number of virus-infected foci and increased lesion size. Thus under two sequential CVB serotype infections, sensitization of both immune systems to a common CVB group antigen could lead to more severe disease.

Protection afforded by sequential CVB infections is hypothesized to occur through binding of nonneutralizing heterotypic antibody to the second virus and promoting clearance of the more virulent virus. Although the authors are not aware of such an example for picornaviruses, studies of nonneutralizing antibodies against alphaviruses, flaviviruses, coronaviruses, orthomyxoviruses, and paramyxoviruses show that such antibodies can provide protection in vivo [29]. Additional studies involving avirulent/virulent CVB which cause myocarditis and other types of disease in mice are clearly warranted to provide further information of future relevance to generation of a CVB vaccine.

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