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Alterations in major histocompatibility complex association of myocarditis induced by coxsackievirus B3 mutants selected with monoclonal antibodies to group A streptococci

(autoimmunity/molecular mimicry)

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ABSTRACT **Three monoclonal antibodies (mAbs), 49.8.9, 36.2.2, and 54.2.8, made to the group A streptococcus M5 serotype identify crossreactive epitopes in cardiac tissues and also neutralize a highly myocarditic variant of coxsackievirus B3 (H3). Mutants of H3 were selected with these mAbs and evaluated for pathogenicity compared with the wild-type virus. H3 and the mutant variants selected with mAbs 36.2.2 (H3-36) and 54.2.8 (H3-54) induced severe myocarditis in DBA/2 (*H-2*^{d}) and A/J (*H-2*^{a}) male mice, whereas CBA (*H-2*^{k}) mice were disease resistant. The virus variant isolated with mAb 49.8.9 (H3-49) was strikingly different and caused disease in CBA and A/J mice but not in DBA/2 animals, suggesting that the major histocompatibility complex association of the disease had been altered. This hypothesis was confirmed by using B10 congenic mice. In addition, T lymphocytes from the H3 and H3-49 virus-infected mice responded to distinctly different peptides in the streptococcal M protein, suggesting that certain epitopes of infectious agents which are shared with host tissues may be critical in determining disease susceptibility in genetically distinct individuals.**

Evidence increasingly implicates virus and microbial infections as triggers of autoimmune diseases (1–3). Antigenic mimicry has been demonstrated between a wide range of infectious agents and normal tissue antigens (4–11), and antibodies to these crossreactive epitopes can often be obtained (7). These autoreactive antibodies frequently have variable effects on disease pathogenicity (7, 8, 11, 12). Some monoclonal antibodies (mAbs) to crossreactive epitopes directly cause tissue injury *in vivo* (7, 11). Other antibodies are poorly pathogenic when given to normal animals but significantly augment tissue injury in the presence of virus (8). Various picornaviruses, including coxsackieviruses, contain mimicking epitopes in their structural proteins to self-molecules in the heart and other organs (8, 10, 11, 13–15). Cunningham *et al.* (16) demonstrated that mAbs identifying shared epitopes between group A streptococcal M5 protein and heart antigens also possessed significant neutralizing activity to coxsackievirus group B type 3 (CVB3). Additionally, one of these mAbs reacted specifically with pathogenic, but not with nonpathogenic, variants of CVB3.

Antibody driven selection can be used to derive variants of highly mutable viruses (12, 17–21). Often, such antibody-selected virus variants are significantly attenuated and can be used to identify regions in the virus genome associated with pathogenicity (12). Previous work showed that the Woodruff strain of CVB3 infected the myocardium of various inbred strains of mice but caused cardiac inflammation only in animals having the *H-2*^{d} haplotype (22). In the present work, we used three anti-streptococcal mAbs to select new CVB3 variants which were evaluated for changes in their ability to induce myocarditis in inbred strains of mice. This study demonstrates that viruses altered by antibodies to crossreactive epitopes shared between an infectious agent and host tissue can substantially shift the genetic susceptibility associations of virus-induced diseases. These epitopes may disturb the interaction between infections and the immune system, resulting in autoimmune destruction in genetically susceptible individuals.

MATERIALS AND METHODS

Mice. Male DBA/2, A/J, CBA/J, C57BL/Sn10J (B10), B10.D2/nJ, B10.A, B10.A(2R), and B10.BR mice 5–7 weeks of age were purchased from Jackson Laboratories, Bar Harbor, ME.

Viruses. A plaque-purified CVB3 variant (H3) was isolated as described (23). Antibody-selected variant viruses were obtained by culturing 10^6 plaque-forming units (pfu) of H3 virus with 100 μg of mAb on HeLa cells. After culture for 20 hr, the HeLa cells were homogenized, mixed with 100 μg of the mAb, and added to fresh HeLa cell cultures. This process (blind passage) was repeated up to two more times until the supernatants produced >50% lysis of HeLa cell monolayers. The new CVB3 variants designated H3-36, H3-49, and H3-54 were derived by using mAbs 36.2.2, 49.8.9 and 54.2.8, respectively, and were plaque purified (23) and purified by ultracentrifugation on sucrose gradients (24).

Antibodies. Production, maintenance, and antigen specificities of murine IgM mAbs to M-type-5 *Streptococcus pyogenes* have been described (25, 26). In brief, mAb 36.2.2 reacts with group A streptococcal M-protein serotypes 1, 5, and 6 and with the following cellular proteins: myosin, tropomyosin, actin, and keratin (25–27), as well as laminin (S. M. Antone and M. W. Cunningham, unpublished work). mAb 49.8.9 reacts with streptococcal M5 and M6 proteins and with vimentin (28). mAb 54.2.8 reacts with streptococcal M5 and M6 proteins and with myosin, tropomyosin, and DNA (25–27). A hyperimmune horse anti-CVB3 was purchased from the American Type Culture Collection. Hybridoma clones GK 1.5 (anti-L3T4), 2.43 (anti-Lyt-2.2), and 30-H12 (anti-Thy-1.2) were purchased from the American Type Culture Collection. mAbs from these clones were isolated as described (29).

Organ Virus Titers and Virus Neutralization Assays. These procedures were performed as described (30).

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Abbreviations: CVB3, coxsackievirus B3; mAb, monoclonal antibody; MHC, major histocompatibility complex; pfu, plaque-forming unit(s).

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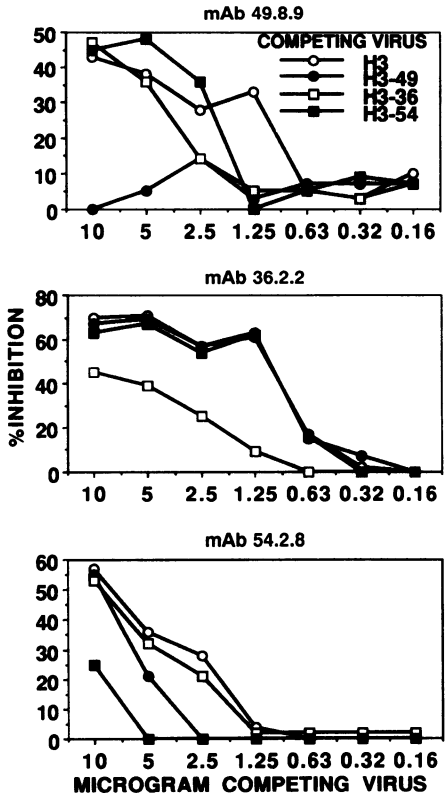

FIG. 1. CVB3 neutralization with streptococcal mAbs. Results represent mean percent reduction in pfu in the presence of the CVB3-inhibitory antibodies compared with virus incubated with equivalent concentrations of normal mouse serum. Each point represents the mean of four replicate cultures.

Competition ELISA. Approximately 10 μg of H3 virus in carbonate buffer (pH 9.6) was dispensed into wells of Pro-Bind microtiter ELISA plates (Becton-Dickinson) and the plates were incubated at 4°C overnight. Approximately 2 μg of mAb was mixed with between 10 and 0.16 μg of soluble sucrose gradient-purified virus (competing virus) and incubated for 45 min at room temperature. The mixture was then added to the H3 virus-coated wells. After incubation for 60 min at room temperature, the wells were washed with phosphate-buffered saline containing 0.05% Tween 20 (Sigma) and the amount of bound antibody was determined by ELISA using urease-conjugated goat anti-mouse IgM antibody (Sigma) and urease substrate (30). Percent inhibition of binding was calculated compared with mAb alone added to the ELISA plate (no competing virus). All results were determined on three replicates per group.

Synthetic Peptides. Overlapping peptides copying the primary structure of the M5 protein (31) were chemically synthesized on a DuPont RAMPS manual peptide synthesizer using the fluorenylmethoxycarbonyl strategy (32). The peptides were synthesized to represent the A, B, and C repeats of the M5 molecule with a 5-aa overlap at either end of the peptide (see Fig. 4; the pepsin cleavage site in M5 protein is marked). The amino acid composition of each HPLC-purified synthetic peptide was confirmed by quantitative amino acid analysis. The peptide designations (and amino acid sequences) are NT1 (AVTRGTINDPQRAKEALD), NT2 (KEALDKYELENHDLKTKN), NT3 (LKTKNEGLKTENEGLKTE), NT4 (GLKTENEGLKTENEGLKTE), NT5 (KKEHEAENDKLKQQRDTL), NT6 (QRDTLSTQKETLEREVQN), NT7 (REVQNTQYNNETLKIKNG), NT8 (KIKNGDLTKELNKTRQEL), B1A (TRQELANKQQESKENEKAL), B1B (ENEKALNELLEKTVKDKI), B1B2 (VKDKIAKEQENKETIGTL), B2 (TIGTLKKILDETVKDKIA), B2B3A (KDKIAKEQENKETIGTLK), B3A (IGTLKKILDETVKDKLAK), B2B3B (DKLAKEQKSKQNIGALKQ), B3B (GALKQELAKKDEANKISD), C1A (NKISDASRKGLRRDLDAS), C1B (DLDASREAKKQLEAEHQK), C1C2 (AEHQKLEEQNKISEASRK), C2A (EASRKGLRRDLDASREAK), C2B (SREAKKQLEAEQQKLEEQ), C2C3 (KLEEQNKISEASRKGLRR), and C3 (KGLRRDLDASREAKKQ).

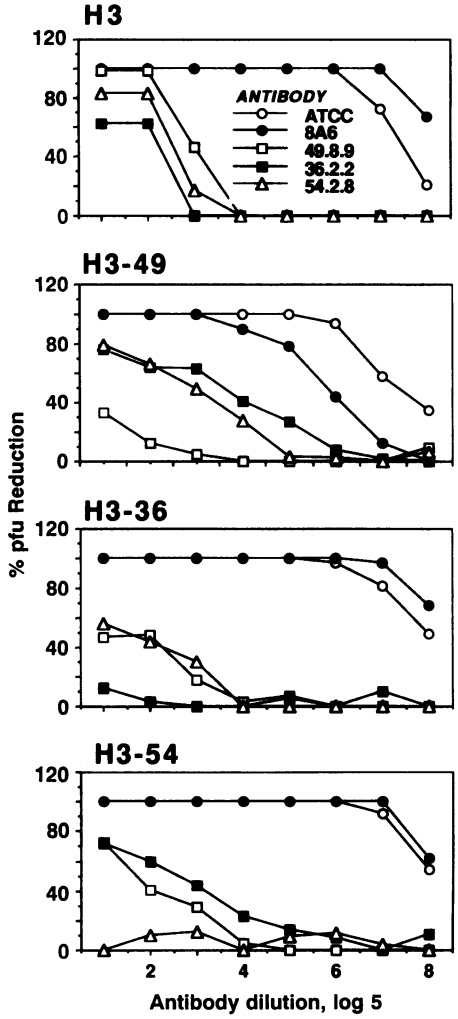

FIG. 2. Competitive inhibition of mAb binding to CVB3. mAbs 49.8.9, 36.2.2, and 54.2.8 were incubated with various concentrations of soluble competing virus and then added to ELISA plates coated with H3 virus. Binding of the mAbs was determined by using urease-conjugated goat anti-mouse IgM antibody. Results represent the mean inhibition of mAb binding compared with wells with the mAb alone. ATCC antibody, horse anti-CVB3.

Lymphocyte Proliferation. Mesenteric lymph node cells were isolated from mice inoculated i.p. with virus at 5×10^4 pfu 7 days earlier, resuspended in Dulbecco's modified

Table 1. Comparison of CVB3 variants *in vivo*

Virus variant	Myocarditis score, mean ± SEM (*n*)		
	DBA/2 (H-2^d)	A/J (H-2^a)	CBA (H-2^k)
H3	1.9 ± 0.3 (13)	1.6 ± 0.2 (11)	1.0 ± 0.2 (12)
H310A1	1.0 ± 0.3* (10)	0.0 ± 0.0 (6)	0.4 ± 0.2 (5)
H3-49	1.3 ± 0.3* (12)	1.6 ± 0.3 (11)	1.5 ± 0.2* (10)
H3-36	1.7 ± 0.3* (7)	2.4 ± 0.2* (7)	1.0 ± 0.3 (6)
H3-54	2.3 ± 0.3 (7)	2.0 ± 0.4 (6)	0.2 ± 0.2 (6)

Values show mean histology score on a 0–4 scale as described (22). Number of animals per group is given in parentheses.
*Value significantly different ($P \leq 0.05$) from H3.

Table 2. Susceptibility of congenic mouse strains to H3 and H3-49 virus-induced myocarditis

Mouse strain	H-2 haplotype				Myocarditis score, mean ± SEM (*n*)	
	K	IA	IE	D	H3	H3-49
C57BL/10	b	b	b	b	0.7 ± 0.5 (4)	0.3 ± 0.2 (6)
B10.D2	d	d	d	d	2.2 ± 0.4* (5)	0.7 ± 0.3 (7)
B10.A	k	k	k	d	2.6 ± 0.2* (5)	1.5 ± 0.3* (4)
B10.A (2R)	k	k	k	b	1.2 ± 0.5 (4)	1.8 ± 0.5* (5)
B10.BR	k	k	k	k	1.3 ± 0.2 (7)	2.6 ± 0.2* (13)

*Value significantly different ($P \leq 0.05$) from C57BL/10.

Eagle's medium containing 10% fetal bovine serum and 20 μM 2-mercaptoethanol, and dispensed (5×10^4 cells per well) into 96-well tissue culture plates together with synthetic streptococcal M5 peptides (10 μg/ml). The plates were incubated at 37°C in a humidified 5% CO_2 incubator for 4 days, labeled with 1 μCi (37 kBq) of [*methyl*-^{3}H]thymidine (51 Ci/mM, ICN) for 20 hr, and harvested onto glass fiber strips. Sections containing radioactivity were placed in vials containing 3 ml of Ecolume scintillation fluid (ICN) for measurement of radioactivity in a Beckman liquid scintillation counter (33).

Histology. Hearts were removed 7 days after infection, fixed in buffered 10% formalin, and stained with hematoxylin and eosin. Sections were coded and blindly scored for myocardial inflammation by either S.A.H. or John Craighead (Department of Pathology, University of Vermont) using a scale of 0–4 (23).

Statistical Analysis. Statistical evaluations were performed by either the Wilcoxon ranked-score test or the Student *t* test.

RESULTS

Selection of Antibody-Escape Mutant Viruses. Fig. 1 shows the ability of H3 virus and the various variant viruses derived from H3 to be neutralized by a standard hyperimmune horse anti-CVB3 serum and mAbs 49.8.9, 36.2.2, and 54.2.8. All viruses were equally inhibited in the neutralization assay by the hyperimmune antibody, confirming that each variant retained important epitopes defining the CVB3 serotype. All three streptococcal M5 protein mAbs neutralized the parent H3 virus, as shown previously (16). However, in each case, the antibody-escape mutant variants were no longer neutralized by the mAb used for their selection but retained the ability to be neutralized by the other two mAbs. Next, competitive inhibition studies determined the ability of the variant viruses to prevent binding of the mAbs to H3 (Fig. 2). Preincubation of any of the three mAbs with soluble H3 significantly abrogated subsequent antibody binding to attached virus in the ELISA. H3-49 virus did not block mAb 49.8.9 binding, nor did H3-36 or H3-54 viruses block mAb 36.2.2 or 54.2.8, respectively. These results imply that important alterations exist in the variant viruses which have changed the availability of the crossreactive epitopes defined by the streptococcal mAbs.

Determining Pathogenicity of the Variant Viruses. If crossreactive epitopes between CVB3 and streptococcal M5 protein identify important epitopes in autoimmune myocardial disease, then the variant viruses in which these epitopes have been changed should show alterations in virus pathogenicity. Previous studies have used congenic strains of mice to map control of the pathogenicity of the Woodruff strain of CVB3 to the D region of the *H-2*^{d} locus (22). To investigate whether major histocompatibility complex (MHC) control of the new variant viruses had changed, male DBA/2J (*H-2*^{d}), A/J (*H-2*^{a}), and CBA/J (*H-2*^{k}) mice were infected with 5×10^4 pfu of the various virus variants and their hearts were evaluated 7 days later for myocarditis (Table 1). H3 virus was highly pathogenic in both DBA/2J and A/J mice but was not pathogenic in CBA animals, even though high cardiac virus titers were observed in all three mouse strains (data not shown). H3-36 and H3-54 viruses showed no apparent phenotypic differences from the parent virus. The H3-49 variant had changed its MHC disease linkage, since it produced myocarditis in A/J and CBA/J but not DBA/2J mice. To confirm the change in MHC association, B10 and B10 congenic mice were infected with either H3 or H3-49 virus and sacrificed 7 days later. Table 2 roughly maps H3 susceptibility to the *H-2D*^{d} region, since B10.A mice developed myocarditis with H3 virus but B10.A(2R) mice were resistant. In contrast, H3-49 virus caused myocarditis in B10.BR(H-2^k), B10.A, and B10.A(2R) mice, suggesting that control of myocarditis susceptibility with this variant may no longer reside in the *H-2D* region. Fig. 3 shows representative histological sections in B10, B10.D2, and B10.BR mice infected with either H3 or H3-49 virus. Generally, H3 virus induced larger and more numerous inflammatory lesions in B10.D2 mice (Fig. 3*B*) than in either B10 or B10.BR mice

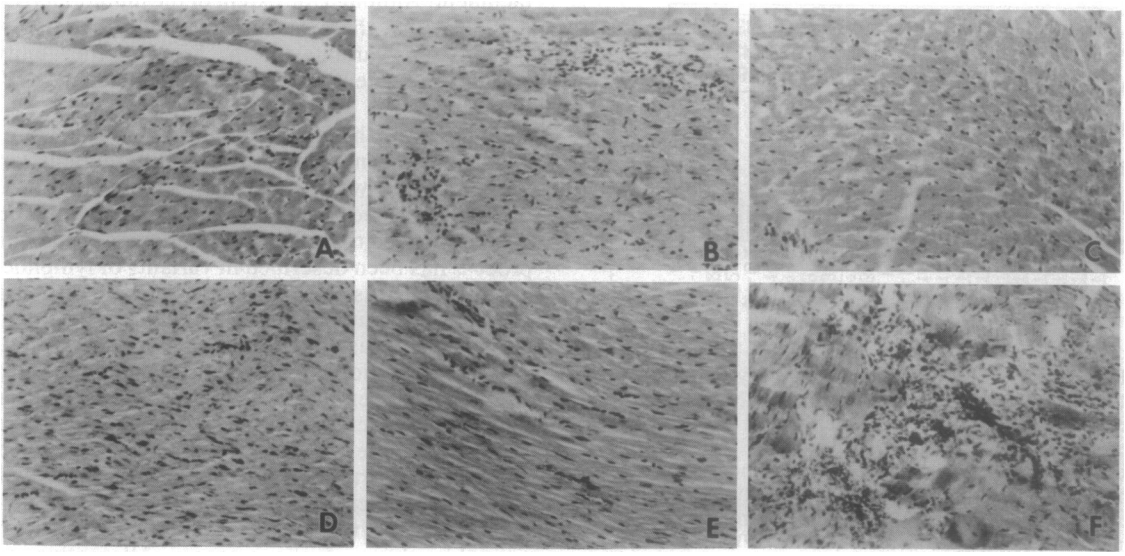

FIG. 3. Hematoxylin- and eosin-stained sections of mouse hearts from B10 (*A* and *D*), B10.D2 (*B* and *E*), and B10.BR (*C* and *F*) mice hearts infected with H3 (*A–C*) or H3-49 (*B–F*) mice. (×165.)

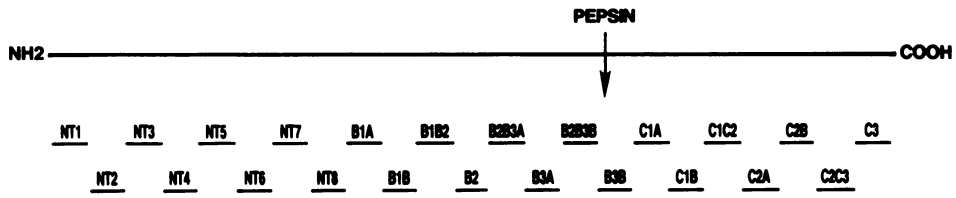

FIG. 4. Diagram representing the group A streptococcal M5 protein and the relative locations of the peptides derived from this protein.

(Fig. 3 *A* and *C*). H3-49 virus produced only scattered lesions usually consisting of <20 inflammatory cells in B10 and B10.D2 mice (Fig. 3 *D* and *E*), whereas lesions were much more extensive in the susceptible B10.BR strain (Fig. 3*F*).

T-Cell Responses in CVB3-Infected Mice to Streptococcal M5 Peptides. Since CVB3 and group A streptococcal M5 protein share crossreactive B-cell epitopes (16) and anti-streptococcal mAbs 49.8.9, 36.2.2, and 54.2.8 demonstrate virus-neutralizing activity, the following experiments were designed to determine whether CVB3 infection of mice also induced T-cell responses to streptococcal M5 peptides. Mesenteric lymph node cells obtained 7 days after infection of B10, B10.D2, and B10.BR mice with either H3 or H3-49 virus were cocultured with overlapping 18-mer synthetic peptides of the streptococcal M5 protein at 10 μg/ml (Fig. 4). Controls consisting of lymphocytes from uninfected mice stimulated with the same peptides showed no significant response above lymphocytes cultured with medium alone (data not shown). The patterns of immune lymphocyte responses differed between virus variants and mouse strains. The H3 virus-immune B10.D2 lymphocytes responded most strongly to peptides NT-2 and NT-6 in the amino-terminal region of the M5 molecule, with NT-6 containing the most reactive amino acid sequence. In the B-repeat region near the center of the M5 molecule, peptides B1–B and B2 were immunodominant in the B10.D2 H3 virus-infected mice. The C-repeat carboxyl-terminal end of the M5 did not produce as much responsiveness as the N-terminal and B-repeat peptides. Lymphocytes from H3 virus-infected B10.BR and B10 mice gave minimal responses to M5 peptides (Fig. 5). In contrast, lymphocytes from B10.BR mice infected with H3-49 virus responded primarily to NT-4. NT-4 is both a B- and a T-cell epitope of M protein that crossreacts with myosin (S. M. Antone and M. W. Cunningham, unpublished observations). Lymphocytes from H3-49 virus-infected B10 and B10.D2 mice give minimal responses to the peptides. Finally, H3 virus-immune B10.D2 lymphocytes were selectively depleted of either all T cells, $CD4^+$ cells, or $CD8^+$ cells and stimulated with the appropriate M5 peptides (Table 3). The proliferation responses to the individual M5 peptides depended upon the presence of either $CD4^+$ or $CD8^+$ T lymphocytes in the cell population.

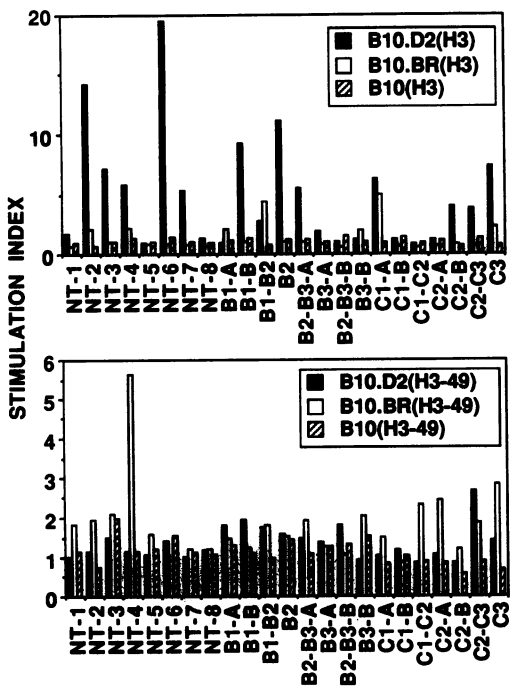

FIG. 5. Proliferation of CVB3-immune lymphocytes in response to M5 peptides. Lymphocytes obtained from B10, B10.D2, or B10.BR mice 7 days after infection with either H3 or H3-49 virus were cocultured with M5 streptococcal peptides (10 μg/ml) for 5 days. Proliferation was determined by [^{3}H]thymidine incorporation and the stimulation index represents (cpm in cultures containing peptides)/(cpm in control cultures). Medium control cultures had 249, 136, and 132 cpm for H3 virus-immune B10, B10.D2, and B10.BR lymphocytes, respectively, and 188, 92, and 89 cpm for H3-49 virus-immune B10, B10.D2, and B10.BR lymphocytes, respectively.

DISCUSSION

Our primary goal was to determine whether altering humoral crossreactive epitopes in CVB3 would change virus pathogenicity. The selection method identified virus variants which could not be neutralized by the anti-streptococcal mAbs. Presumably, significant mutational changes occurred in the derivation of H3-49 virus which changed the antibody binding site(s) in the capsid proteins of the virus. The precise location of the mutational changes is not known but may have been either in the antibody binding site itself or in distal portions of the capsid proteins. In the latter instance, mutations causing shifts in the tertiary structure of the virus capsid could disrupt the availability of the binding site without actually mutating the amino acids involved in antibody attachment. Although the extent of the mutation(s) is unknown, it is clear that the mutations did not affect either the infectivity of the variants for the heart or the ability of these variants to be neutralized by a polyclonal anti-CVB3 antiserum.

Potentially, the immunological history of an individual could support antigenic drift in highly mutable viruses. Any virus variants which arise as random mutations and are poorly controlled by the immune response might persist and be shed longer than the initial infecting virus variant. This process has been described in variants of lymphocytic choriomeningitis virus (34) and may be partially responsible for the genetic variability of human immunodeficiency virus and influenza A virus (7, 21, 35).

CVB3 infection stimulates T-lymphocyte responses to streptococcal peptides. This implies that molecular mimicry between the bacteria and virus occurs at both humoral and cellular immune levels, and supports the hypothesis that bacteria and viruses share similar structures with host tissues. Crossreactivity between group A streptococci and CVB3 may be the basis for inflammatory heart diseases caused by these infectious agents. Alternative explanations for the T-cell responses to streptococcal epitopes in CVB3 infected mice are equally possible. CVB3 infection may cause

Table 3. T-cell proliferation responses to streptococcal M5 peptides

Antibody treatment	[^{3}H]Thymidine incorporation, mean cpm ± SEM					
	Medium	NT2	NT3	NT4	NT6	NT7
None	165 ± 21	3265 ± 128	1632 ± 222	2007 ± 67	3842 ± 360	2642 ± 399
Anti-Thy-1.2 + C′	237 ± 34	375 ± 23*	257 ± 30*	170 ± 15*	200 ± 37*	145 ± 23*
Anti-L3T4 + C′ (CD4$^+$)	227 ± 48	245 ± 35*	175 ± 13*	175 ± 36*	4215 ± 487	2082 ± 319
Anti-Lyt-2 + C′ (CD8$^+$)	290 ± 57	3848 ± 489	2600 ± 217	1817 ± 15	645 ± 77*	370 ± 46*

Mesenteric lymph node cells from H3 virus-immune B10.D2 mice were untreated or treated with antibody and complement (C′), stimulated with M5 peptides, and labeled with [^{3}H]thymidine. Results represent mean cpm ± SEM of four replicate cultures.
*Value significantly reduced ($P \leq 0.05$) compared with no antibody treatment.

nonspecific polyclonal T-cell activation in response to M5 peptides. This explanation (polyclonal T-cell activation) seems unlikely for several reasons. First, B10 and B10.BR mice infected with H3 virus fail to give vigorous M5 peptide responses, although high cardiac virus titers are present in these animals and B10.D2 mice infected with the same virus give significant T-cell reactions to these peptides. Thus, infection of the heart alone does not trigger crossreactive T-cell responses. A second explanation for immunity to M5 peptides in CVB3-infected animals is that myocyte lysis, resulting from either virus infection or lymphocyte infiltration of the myocardium, releases large quantities of cardiac proteins, including myosin. Since the M5 protein is known to share crossreactive peptides with myosin (25–27, 36), T-cell responses to the myosin released during virus infection might produce the cellular immunity to M5 peptides seen in CVB3-immune lymphocyte populations. Presumably, cloning the M5 peptide-reactive T cells from CVB3-infected animals will be necessary to establish crossreactivity of T-cell epitopes between the bacteria and virus and to identify the potential role of these T cells in autoimmune myocarditis.

In conclusion, these studies demonstrate that at least one crossreactive epitope is shared by the heart, CVB3, and streptococcal M5 protein which may play an important part in the pathogenesis of autoimmune inflammatory heart disease. The significance of this observation may be that very different infectious etiological agents may cause specific diseases by very similar mechanisms. Furthermore, one might speculate that sequential exposure of individuals to various infectious agents possessing similar crossreactive epitopes might lead to periodic reactivation of autoimmunity.

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