HEART-SPECIFIC AUTOANTIBODIES FOLLOWING MURINE COXSACKIEVIRUS B_3 MYOCARDITIS

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The presence of heart-specific autoantibodies in the sera of some patients with Coxsackievirus B_3 (CB_3)[1]-induced myocarditis (1, 2) has suggested that autoimmunity is a sequela of viral myocarditis. Similar heart-specific autoantibodies have previously been found in patients with poststreptococccal rheumatic fever (3), Chagas' disease (4, 5), postpericardiotomy syndrome (6, 7) and post–myocardial infarction syndrome (6, 7). However, the human disease does not easily lend itself to study due to the rarity of the disease and the unknown time course of the original viral infection. Therefore, an animal model is extremely valuable.

Several murine models of myocarditis have been developed to investigate the pathogenic mechanisms involved in CB_3-induced myocarditis. One model described by the Woodruffs (8) has allowed for the study of cytotoxic T lymphocytes (CTL) that are generated in response to the viral infection. It has been demonstrated (9, 10) that these CTL can damage myocardial cells in vitro. These studies have suggested that this cell-mediated response is a major pathogenic mechanism in CB_3 myocarditis in adult male BALB/c mice. Huber and Lodge (11) have found that two populations of CTL are produced during this disease in mice. One population is virus-specific, and preferentially lyses infected myocytes, while the second preferentially lyses uninfected myocytes. This second "autoreactive" CTL population indicates that cellular autoimmunity may play an important role in the development of myocarditis. A second model was developed (12), and was used for the identification of factors that may influence the severity of the disease, such as drug therapy, age, and exercise. The second model differs from the first in the strains and age of the mice used; sex does not appear to be influential. Furthermore, the CB_3 infection resulted in chronic, dilatational heart disease similar to that seen in humans. This model of CB_3-induced myocarditis was the starting point of this study.

Here, we report the discovery of heart-specific autoantibodies in suckling mice following infection with CB_3, corresponding with the development of myocarditis. This finding provides an opportunity to study the development and rele-

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[1] *Abbreviations used in this paper:* AFA, antifibrillary antibodies; ANA, antinuclear antibodies; ASA, antisarcolemmal antibodies; ASMA, anti–smooth muscle antibodies; CB_3, Coxsackievirus B_3; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex.

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vance of these heart-specific autoantibodies in myocarditis and to delineate the corresponding cardiac antigen(s).

Materials and Methods

Infection and Necropsy. 2-wk-old female A.SW/SnJ (*H-2*^{s}) mice (The Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with 0.1 ml 10^5 tissue-infective dose$_{50}$ ($TCID_{50}$) CB_3 (Nancy strain). The mice were killed by retroorbital bleeding at days 2, 3, 5, 7, 9, 15, 21, and 45 postinfection. The serum from individual animals was collected and stored at −70°C until tested. Control animal sera were prepared similarly, except that the animals were inoculated with an uninfected Vero monkey kidney cell culture (Flow Laboratories, Inc., McLean, VA) lysate identical to the culture used to prepare the virus.

Histology. The hearts were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin-eosin according to standard procedures. The slides were then examined for evidence of myocarditis.

Indirect Immunofluorescence. The method used is that of Bigazzi and Rose (13). The heart, kidney, liver, and stomach were removed from an uninfected animal, frozen, and 4 μm sections were cut in a cryotome. The sections were overlaid with a 1:10 dilution of mouse serum, and incubated at room temperature for 30 min. The slides were rinsed, and washed for 30 min in phosphate-buffered saline, pH 7.2. Next, the sections were incubated 30 min at room temperature with either fluorescein isothiocyanate (FITC)-conjugated goat anti–mouse Ig (Cappel Laboratories, Cochranville, PA), FITC-conjugated goat anti–mouse IgG (Tago Inc., Burlingame, CA), or FITC-conjugated goat anti–mouse IgM (Cappel Laboratories). The slides were then rinsed, and washed for 30 min in PBS. Following this final wash, the sections were overlaid with 90% (vol/vol) glycerol/PBS, coverslipped, and examined using a Zeiss fluorescent microscope.

Absorption Studies. Organs were prepared according to a modification of McCabe et al. (14). Briefly, the heart, kidney, liver, and skeletal muscle (gastrocnemius) from 30–50 mice of various strains were rinsed free of blood in cold borate-buffered saline, pH 8.0. The organs were homogenized, centrifuged at 200 *g* for 10 min, and washed until the supernatant was clarified. The pellet was saved as the insoluble fraction. The initial supernatant and all washes were pooled and centrifuged at 16,300 *g* at 4°C for 1 h. The pellet from the high speed spin was saved as the microsomal fraction, and the supernatant was designated the soluble fraction. Each of the three fractions from the four organs was dialyzed, lyophilized, and then mixed (4 mg) with 0.1 ml of diluted (1:10) serum. The mixture was incubated for 1 h at room temperature. Following incubation, the mixture was centrifuged for 10 min in a microfuge. The supernatant was removed and used in an indirect immunofluorescence assay, as described above.

Results

The sera from 59 A.SW mice that had been infected with CB_3 were tested on normal mouse tissues in indirect immunofluorescence assays. Sera taken at days 2 and 3 after infection were not tested due to lingering virus presence. Infected and control animal sera were sampled at days 5, 7, 9, 15, 21, and 45. At days 15, 21, and 45 after infection, heart-reactive autoantibodies could be detected in the animals, in various degrees of incidence and titer. No heart-reactive antibodies were found in any animals before day 15 after infection. All control animals were negative for heart-reactive antibodies. The prevalence of the antibodies decreased from day 15 to day 45. Heart-reactive antibodies were found in 78% (7 of 9) of the animals at day 15, in 50% (4 of 8) of the animals at day 21, and in 40% (4 of 10) of the animals at day 45 postinfection. Although the incidence decreased, there was, among animals that developed antibodies, a

trend toward higher titer; from 30 ± 9 (mean ± SE) at day 15, to 28 ± 8 at day 21, and 85 ± 29 at day 45. All of the antibodies seen were of the IgG isotype. No IgM antibodies were identified in any of the heart-reactive sera. This may be due either to the possibility that the IgM antibodies appeared briefly between our sample times of days 9 and 15, or that they are all absorbed out by the heart of the donor animal. Thus, A.SW mice will produce myocardial autoantibodies as a sequela to CB_3 infection.

Several patterns of immunofluorescent localization could be distinguished with the sera from the CB_3-infected animals (Fig. 1). The first pattern delineated was a reaction with the myocyte membranes (Fig. 1 *A*). These antibodies have been termed antisarcolemmal antibodies (ASA) and were similar to those described in human postviral myocarditis (1, 2), rheumatic carditis (3), postpericardiotomy (6, 7), and post–myocardial infarction (6, 7) syndromes. All 15 animals positive for heart-reactive antibodies demonstrated this reaction. The second reaction was with one or more of the intracellular contractile proteins, which may include actin and myosin (Fig. 1, *B* and *C*). These autoantibodies may be similar to the antifibrillary antibodies (AFA) as described by Maisch et al. (1) in postviral myocarditis. In the sera from animals with ASA, 8 of 15 were also positive for these antibodies.

An additional reaction was seen only at day 45 in three of four animals that displayed the myocardial autoantibodies. These three sera had a focal reaction with kidney tubules (Fig. 1 *D*). Maisch et al. (2) have described a heterologous renal tubular pattern that was attributed to heterophile antibodies. Non–organ-specific autoantibodies, such as anti–smooth muscle antibodies (ASMA) and antinuclear antibodies (ANA) were also observed. The ASMA were found in 8 of 15 of the animals with heart antibodies, and in 5 of 12 of the animals without heart antibodies. No ASMA were detected in the 15 control animals tested. Only one of the infected animals demonstrated ANA, and it was negative for the heart-reactive antibodies. However, 5 of the 15 control animals were positive for ANA. Reactivity with skeletal muscle was present in 3 of 12 antimyocardial antibody–negative sera, and in 12 of 15 antimyocardial antibody–positive sera. Titration experiments showed that this latter reaction was generally one- to twofold weaker in titer than that with heart muscle. The titration results from several animals at the various sample times are shown in Table I. Therefore, A.SW animals develop both organ-specific as well as non–organ-specific autoantibodies after infection with CB_3.

Absorptions were done in an effort to determine whether the autoantibodies were indeed heart-specific, as well as to identify whether soluble or insoluble antigens were involved. Soluble and insoluble fractions were prepared from heart, skeletal muscle, kidney, and liver. Sera were absorbed with individual fractions from each of the organs, as well as a mixture of the fractions. Results from a representative absorption experiment are given in Table II. These results were obtained by absorbing separate samples of the serum with each insoluble organ fraction. The insoluble fraction of the heart homogenate was able to absorb out all reactivity from the sera with the heart, skeletal muscle, and kidney. The microsomal fraction and the soluble fraction of heart only diminished the heart reaction slightly, and had little or no effect on the skeletal muscle or kidney

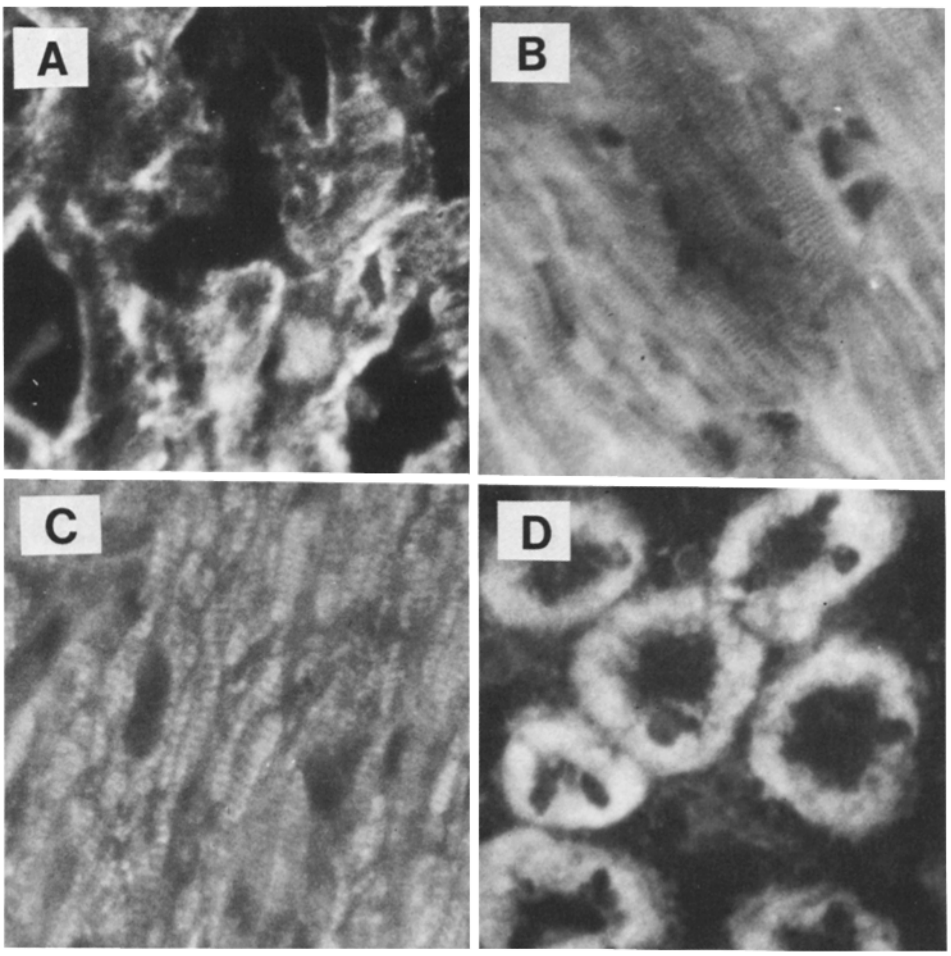

FIGURE 1. Indirect immunofluorescent staining of normal murine heart and kidney tissues with sera from A.SW/SnJ animals 15 and 45 d after infection with CB_3. (*A*) Reactivity with myocyte membrane was observed in a serum that was diluted 1:10. The serum used was obtained from animal 6, which was killed 15 d after infection. This reaction pattern is representative of ASA. (*B*) A serum that was diluted 1:40 was obtained from animal 3 at 15 d after infection. The pattern depicted was that of a fine striated contractile band reaction observed in heart tissue. (*C*) A wider striation band reaction was seen with serum (diluted 1:10) from animal 1, 45 d after CB_3 infection. Reactions seen in *B* and *C* have been collectively termed AFA, since they appear to be reactive with one or more of the intracellular contractile proteins. (*D*) A focal kidney tubule reaction was found in the serum from the same animal (1) shown in *C*.

reactivity. As with the heart, the primary reacting antigen(s) in the skeletal muscle and kidney could be found in the insoluble fraction. The kidney insoluble fraction could only absorb out the kidney reaction. The skeletal muscle insoluble fraction totally removed all skeletal muscle reactivity, but only slightly diminished the antimyocardial titer.

All of the sera that demonstrated some reactivity to heart were absorbed with the skeletal muscle insoluble fraction. The results of these absorptions are found

TABLE I
Titration of A.SW Autoantibodies on Normal Tissue

Days after infection	Animal	Antibody titer*					
		Heart		Skeletal Muscle		Kidney	Liver
		ASA	AFA	ASA	AFA		
15	1	20	0	0	0	0	0
	2	20	20	0	0	0	0
	3	40	40	20	10	0	0
	4	80	80	40	0	0	0
	5	20	0	20	10	0	0
	6	20	0	10	0	0	0
	10	10	0	10	0	0	0
21	2	40	40	40	40	0	0
	7	10	10	10	0	0	0
	8	40	40	20	20	0	0
	9	20	20	20	20	0	0
45	1	80	80	40	40	80	0
	5	20	20	10	10	0	0
	8	80	80	80	80	80	0
	10	160	160	40	40	160	0

* The antibody titer is expressed as the reciprocal of last dilution that shows a positive reaction.

TABLE II
Absorption of Autoantibodies with the Insoluble Fraction of Organ Homogenates*

Absorbing organ	Tissue reaction‡					
	Heart		Skeletal Muscle		Kidney	Liver
	ASA	AFA	ASA	AFA		
Unabsorbed	4	4	3	2	3	0
Liver	4	4	3	2	3	0
Kidney	4	4	3	2	0	0
Skeletal Muscle	2	2	0	0	3	0
Heart	0	0	0	0	0	0

* Serum, obtained from A.SW animal 8 45 d after infection, was used at 1:10 dilution.
‡ Tissue reaction was determined by indirect immunofluorescence. The intensity of fluorescence observed was graded on a 0–4 scale.

in Table III. Several sera that reacted only with skeletal muscle were also absorbed in a similar manner. In all but one serum, the skeletal muscle fraction was able to remove the skeletal muscle antibodies while not removing or only slightly diminishing the heart reaction. Absorption with the skeletal muscle fraction had no effect on the antibodies that reacted with kidney. However, there were various effects on the anti-smooth muscle antibodies in these sera. In some sera, the ASMA were only lessened, while in other sera, the ASMA could be completely removed. To make certain that the inability of the skeletal muscle fraction to remove the heart reaction was not simply a quantitative difference,

TABLE III

Absorption of Autoimmune Sera with the Insoluble Fraction of Skeletal Muscle

Days after infection	Animal	Tissue reaction*					
		Heart		Skeletal Muscle		Kidney	Smooth muscle
		ASA	AFA	ASA	AFA		
15	1	2 (1)‡	1 (1)	2 (0)	1 (0)	0	0
	2	2 (1)	2 (1)	1 (0)	1 (0)	0	1 (0)
	3	3 (3)	3 (3)	3 (0)	3 (0)	0	1 (1)
	4	4 (2)	4 (2)	2 (0)	1 (0)	0	1 (0)
	5	3 (2)	3 (2)	1 (1)	1 (1)	0	2 (0)
	6	3 (2)	3 (2)	2 (0)	2 (0)	0	3 (2)
	7	2 (0)	2 (0)	2 (0)	1 (0)	0	1 (1)
	9	2 (0)	2 (0)	1 (0)	1 (0)	0	3 (2)
	10	2 (1)	2 (1)	2 (0)	2 (0)	0	2 (0)
21	1	2 (0)	1 (0)	2 (0)	2 (0)	0	1 (1)
	7	1 (1)	1 (1)	0	1 (0)	0	0
	8	2 (1)	2 (1)	3 (0)	2 (0)	0	0
	9	3 (3)	3 (3)	2 (0)	2 (0)	0	2 (1)
	10	1 (0)	1 (0)	1 (0)	0	0	0
45	1	4 (3)	4 (3)	3 (0)	3 (0)	2 (2)	0
	3	2 (0)	0	2 (0)	1 (0)	0	1 (0)
	5	2 (2)	2 (1)	1 (0)	2 (0)	0	3 (1)
	6	2 (0)	1 (0)	2 (0)	2 (0)	0	0
	8	3 (2)	3 (2)	3 (0)	2 (0)	3 (3)	0
	10	3 (2)	2 (2)	3 (0)	1 (0)	2 (3)	0

* Tissue reaction was determined by indirect immunofluorescence and the intensity of fluorescence observed was graded on a 0–4 scale.

‡ The first score represents the tissue reactivity before absorption and the score in parenthesis represents the tissue reactivity after absorption.

the absorption with the skeletal muscle fraction was repeated sequentially three times. No further decrease in the heart reaction was noticed. The liver fractions had no effect on any of the reactions, and therefore served as a negative control. Thus, these experiments demonstrated that the heart-reactive antibodies were indeed heart-specific, and distinct from the antibodies that crossreacted with kidney and skeletal muscle.

The autoantibody-containing sera were also tested on normal rat and baboon tissues. The reactions identical to those in mouse tissue were found with rat and baboon heart and skeletal muscle. However, the heterogeneous kidney tubule reaction was found with the rat kidney, but not with the baboon kidney. The absorptions with the murine organ fractions also removed the reactions against the rat and baboon tissues. Therefore, these heart autoantibodies are organ-specific, but neither heterophile in nature, nor species specific. It appeared that the skeletal muscle crossreaction was also not species specific, while the kidney tubule reaction was rodent specific.

Pathological findings in the A.SW hearts after day 9 consisted of large focal lesions containing lymphocytes, polymorphonuclear neutrophils, and macrophages. In addition, an interstitial infiltrate composed of large and small mono-

nuclear cells was observed. All of the 15 animals that demonstrated these heart-specific autoantibodies also had some evidence of myocardial damage. These animals showed evidence of an ongoing inflammatory process, with the peak occurring at day 15. As determined by an analysis of variance test, there was no correlation between the severity of the myocardial involvement at the time of necropsy and the presence, titer, and/or reactivity pattern of the autoantibodies, as shown in Table IV. Therefore, these autoantibodies indicated that myocardial damage had occurred, but neither their presence, reactivity, nor titer indicated the degree of damage at the time they were found.

Discussion

We describe the first animal model of post-CB_3 myocarditis where heart-specific autoantibodies are produced. Similar autoantibodies have been described by Maisch and coworkers (1, 2) in patients with post-CB myocarditis. The autoantibodies appear to be directed mainly against the membrane or a membrane-bound antigen. Further work in our laboratory is underway to identify the antigen(s) against which these autoantibodies are directed. Whether or not these heart-specific autoantibodies are mediators of damage remains to be determined. We have examined other mouse strains, in addition to the A.SW inbred strain used in this study (Wolfgram, Beisel, Herskowitz, and Rose, submitted for publication). Heart-specific autoantibodies were found in all four of the congenic strains with the A background, regardless of their *H-2* haplotype, and all developed the typical ongoing myocarditis. In contrast, none of the B10

TABLE IV

Correlation of the Autoantibody Titer with Severity of Myocardial Lesions

Days after infection	Animal	Heart-specific antibody titer	Pathological index*
15	1	20/0‡	1.2
	2	20	1.0
	3	40	3.8
	4	80	2.5
	5	20/0	1.5
	6	20/0	2.2
	10	40	3.0
21	2	40	1.0
	7	10	1.0
	8	40	1.8
	9	20	3.8
45	1	80	0.8
	5	20	1.0
	8	80	2.5
	10	160	0.5

* The pathological index is a mean of the scores (0–4) given by two independent observers.

‡ The titer of ASA/AFA reactivity. If the serum had identical titers for both reaction patterns, then only one titer is given.

congenics produced these autoantibodies, and none showed chronic myocardial inflammation. Besides the non–MHC (major histocompatibility complex)–regulated predisposition, an MHC influence was noted in the observed variation of incidence and autoantibody titer among the four A-background *H-2* congenics. Further investigations will determine the genetic parameters that control the predisposition to the production of these heart-specific autoantibodies and to ongoing myocarditis.

Correlation between an infectious disease and the induction of an autoimmune disease has been investigated in several systems (14, 15). The possibility of a viral etiologic agent has been postulated in diseases such as juvenile diabetes mellitus (15) and postmeasles encephalitis (16). However, proving that a virus is the primary etiologic agent is a difficult task in the human situation because viral isolations are rare and, usually, the only connection is the clinical history and rising antiviral antibody titers. Therefore, a suitable animal model will prove to be crucial for establishing a causative relationship between autoimmunity and viral myocarditis.

There are several current hypotheses that could explain the development of these antibodies. First, the immunofluorescent reaction with frozen heart tissue indicates that some normal heart antigen(s) may be the target. Infection and/or inflammation may render heart components antigenic. This situation has been hypothesized by Szarfman et al. (4) in Chagas' disease following infection with *Trypanosoma cruzi.* A second hypothesis is that antiviral antibodies elicited by CB_3 may crossreact with a normal heart antigen. A similar situation has been found in poststreptococcal rheumatic fever (3, 17), and has been reported (5) in Chagas' disease. A third hypothesis is that CB_3 induces a myocardial neoantigen (18) to which antibodies are produced. Our finding that the autoantibodies react with normal heart tissue makes this possibility less likely. A more recent theory holds that autoantibodies may be antiidiotypic antibodies against antiviral antibodies that may react with the viral receptor (19, 20). All of these possibilities are currently under examination.

Since our demonstration of autoantibodies against cardiac tissue indicates the development of an autoimmune process, the question of autoimmunity and its importance in the pathogenesis of virally induced myocardial injury must be raised. Potentially, autoimmunity can develop in genetically predisposed individuals whenever damage is done to the cardiac tissue. This can occur with a bacterial, viral, or parasitic infection, after open-heart surgery, or after a myocardial infarction. Therefore, this murine model CB_3-induced myocarditis can be used to investigate the genetic predisposition, cellular, and humoral mechanisms, and perhaps possibilities for treatment for cardiac autoimmunity in postviral myocarditis. The first step necessary in these investigations is to isolate the antigenic target and demonstrate that it is capable of initiating an immunopathological reaction. The availability of autoantibodies in postinfection sera will permit us to identify the antigen(s) responsible.

Summary

The sera from A.SW/SnJ mice infected with Coxsackievirus B_3 (CB_3) were tested on normal mouse tissue by indirect immunofluorescence. Heart-reactive

antibodies were found. Absorption studies with organ extracts showed some of these autoantibodies to be heart-specific. Additional antibodies were crossreactive with skeletal muscle and kidney. These findings suggest a role for autoimmunity in the pathogenesis of murine CB_3-induced myocarditis. This study establishes an animal model for the study of the humoral autoimmune response in human viral myocarditis.

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