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Coxsackievirus induced myocarditis in mice: cardiac myosin autoantibodies do not cross-react with the virus

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SUMMARY

After infection with Coxsackievirus B_3 (CB_3), *H-2* congenic strains of mice on an A-background develop an immunologically mediated myocarditis associated with autoantibodies directed mainly against cardiac myosin. We tested these autoantibodies for cross-reactivity with the virus. Using immunoblotting and virus neutralizing assays with affinity purified antibodies and absorbed sera, we found that the autoantibodies to heart myosin did not cross react with CB_3. In addition, myosin autoantibodies induced by immunization with cardiac myosin did not react with the virus. Western immunoblotting revealed that the anti-CB_3 antibodies in the infected mice are directed against the capsid protein VP 2. We conclude that CB_3 antigens do not stimulate an immune response that cross-reacts with cardiac myosin.

Keywords myocarditis Coxsackievirus B_3 myosin autoimmunity

INTRODUCTION

Several human diseases, including multiple sclerosis (Lisak, 1980; Waksman & Reynolds, 1984), post-measles encephalitis (ter Meulen, Katz & Müller, 1972; Johnson *et al.*, 1984) and insulin-dependent diabetes mellitus (Rossini, Mordes & Like, 1985) have been attributed to post-infectious autoimmunity. An autoimmune component is also observed in inflammatory heart diseases after infection with certain streptococcal strains (Kaplan & Svec, 1964) and various viruses (Maisch *et al.*, 1982). Several theories have been proposed to explain how post-infectious autoimmunity is triggered. One theory suggests that the infectious agent carries epitopes that cross-react with normal host cell components, thereby initiating an autoimmune response. Experimental evidence for this view comes from the presence in patients with rheumatic fever of heart-reactive autoantibodies that crossreact with certain streptococcal proteins (Zabriskie, Hsu & Seegal, 1970; van de Rijn, Zabriskie & McCarty, 1977). Another theory is that the tissue destruction caused by the infectious agent results in release or exposure of intracellular autoantigens. This theory could explain the heart-autoreactivity which has often been observed after cardiac injury in clinical and experimental situations (Kuch, 1973; Maisch, Berg & Kochsiek, 1979).

To investigate the mechanism of immunologically mediated myocarditis following Coxsackievirus B_3 (CB_3) infection, several murine models have been developed (Lerner & Wilson, 1973; Woodruff, 1980; Beisel *et al.*, 1985). In particular, Wolfgram *et al.* (1986) have identified A *H-2*

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congenic strains of mice which develop two peaks of myocardial lesions following CB_3 infection. The first peak is most probably related directly to the viral infection and the consequent inflammatory response. A second, later phase of the disease is characterized by a diffuse mononuclear cell infiltrate and by the presence of heart specific autoantibodies and can, therefore, be related to autoimmunity. Using immunochemical analyses, Alvarez *et al.* (1987) found that the heavy chain of myosin is the major antigen recognized by the heart-specific autoantibodies in mice genetically predisposed to develop CB_3 induced autoimmune myocarditis. Subsequently, Neu *et al.* (1987) demonstrated that the autoimmune response is specific for the cardiac isoform of this molecule.

The identification of the major autoantigen provides us with the opportunity to address the question of whether there are antigens shared by cardiac myosin and the virus that might initiate the autoimmune myocarditis. The results of this investigation are reported here.

MATERIALS AND METHODS

Infection. CB_3 (Nancy Strain), a myocarditic variant in mice, was grown in Vero monkey kidney cells and was originally obtained from Drs Lerner and Khatib, Wayne State University, Detroit, MI. The virus titration and virus preparation for infection has been described (Wolfgram, Beisel & Rose 1986). Two-week-old A.CA/SnJ inbred mice were inoculated intraperitoneally with 0·1 ml of 10^5 $TCID_{50}$ of CB_3 as described by Wolfgram *et al.* (1986). Serum was obtained by retroorbital bleeding 21 days after infection, three different serum pools were made and stored at −70°C. Normal mouse serum (NMS) was obtained from 5-week-old uninfected mice.

Isolation and purification of cardiac myosin. Murine cardiac myosin was isolated from mouse hearts according to the method of Kielley & Harrington (1960) with some modifications. Briefly, 40 hearts were rinsed in cold phosphate buffered saline (PBS), pH 7·2, transferred to 10 vol of 4°C extracting solution (0·1 M K_2HPO_4, 0·5 M KCl) containing 20 μl of 0·2 M phenylmethylsulphonyl fluoride (PMSF) in DMSO/10 ml. The hearts were then minced with scissors and homogenized in a conical ground glass homogenizer and pestle. The suspension was extracted for 10 min on ice, with mixing, and then centrifuged at 48,000 ***g*** (20,000 rev/min in Sorvall SS 34) for 30 min. The supernatant contains the myosin. The pellet was re-extracted with 5 vol of extracting solution; the volume of the supernatants was measured and adjusted to pH 6·6 with 0·5 M CH_3COOH. Ten volumes of ice-cold distilled water were added slowly, with stirring, to precipitate the myosin along with any extracted actin. The pH was checked, and readjusted to 6·6 if necessary. The precipitate was collected by centrifuging at 30,000 ***g*** for 20 min (16,000 rev/min in SS 34 rotor) and dissolved in 12 ml of cold KI-ATP buffer (0·6 M KI, 5 mM ATP, 5 mM dithiothreitol, 1 mM $MgCl_2$, 20 mM imidazole, pH 7). At this point the crude myosin preparation was further purified by ammonium sulphate fractionation and chromatography on a 2·6 × 90 cm column of Biorad A-15 m resin as described for human platelet myosin (Pollard, 1982). The myosin-containing fraction was precipitated with an equal volume of saturated ammonium sulphate, 10 mM EDTA, pH 8·0. The purity of the preparations was checked by sodiumdodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

Preparation of enriched virus. Ten thousand $TCID_{50}$ of CB_3 were added to confluent Vero monkey kidney cells grown in 75 cm^2 tissue culture flasks. The cultures were incubated for 24 h at 37°C or until a cytopathic effect was seen. The remaining adherent cells were scraped off and harvested by centrifugation. They were then washed in PBS, resedimented, and resuspended in a small volume of PBS. The suspension underwent three freeze-thaw cycles to release intracellular virus and was then centrifuged to remove the cell debris. The virus was enriched by the method of Öberg & Philipson (1969). In brief, the sample was overlaid over CsCl (1·4 g/ml) and centrifuged at 51,000 ***g*** in a swinging bucket rotor (SW 39) for 3 h. The virus-rich band was collected after puncture of the tube, dialysed against PBS and stored at −70°C.

Preparation of total proteins from mouse hearts. Preparation of crude heart extracts was performed according to Pardo, Pittenger & Craig (1983) with modifications described by Alvarez *et al.* (1987).

SDS-PAGE and electroblotting. A fresh suspension of the SDS-soluble heart proteins was mixed with an equal volume of 2 × gel sample buffer (10% SDS, 0·5 M Tris-HCl, 25% glycerol, 0·5% bromophenol blue, 1 mM DTT, pH 6·8). Alternatively, four volumes of the virus-enriched suspension were mixed with 1 volume of 5 × sample buffer. The mixtures were boiled for 5 min, centrifuged in a Beckman airfuge for 5 min at 120,000 *g* and then loaded onto the gels. SDS-PAGE was performed in a mini-gel system (Hoeffer Sci. Inst., CA) as described by Alvarez *et al.* (1987). The gels were electroblotted onto 0·45 μm nitrocellulose paper (BA-85, Schleicher & Schuell) as described by Alvarez *et al.* (1987). Then the nitrocellulose paper was cut into strips according to the appropriate lanes on the gel. The strips were either used for immunolabelling or stained for blotted proteins using the Bio-Rad Biotin-Blot Protein Detection Kit according to the manufacturer's instructions.

Dot-blot. Nitrocellulose paper was cut into 1 × 4 cm strips, soaked with 0·1 × PBS and airdried for 15 min at room temperature. Myosin (1 μg in 1 μl of 50 mM sodium pyrophosphate, pH 7·2) and 1 μl of the virus-enriched suspension were applied onto separate spots of the nitrocellulose strip. As control antigen, 1 μl of a Vero monkey kidney cell lysate (obtained from a 1% cell suspension) was used. Then the strips were airdried for 30 min, and used immediately or stored at 4°C over Drierite. One representative strip was stained for proteins as described above.

Immunolabelling of blotted proteins. Immunolabelling was by a peroxidase system as described by Tamashiro *et al.* (1985). Briefly, nitrocellulose strips were placed in 75 × 10 mm glass tubes and blocked with PBS-T (15 mM PBS, 0·1% Tween 20, pH 7·2) containing 2% BSA and 2% normal goat serum for 1 h. Then the blocking solution was decanted and the strips were incubated with the test sera diluted in PBS-T and 1% BSA for 1 h at room temperature. After three rinses and two 10 min washes in PBS-T, the strips were incubated with conjugate solution consisting of 5 μg/ml peroxidase labelled goat anti-mouse Ig (Cappel) in PBS-T and 1% BSA. After 45 min the strips were rinsed three times and washed twice in PBS-T for 10 min, followed by three rinses and two 10 min washes in 0·1 × PBS without Tween. Staining of bound antibody was performed by incubating the strips in chloronapthol solution (10 mg of 4-chloro-1-napthol dissolved in 4 ml methanol and 16 ml 0·1 × PBS and 20 μl 30% H_2O_2) for 20 min. Reactions were stopped with an excess amount of 50 mM Tris/200 mM NaCl, pH 7.

Neutralizing antibody tests. Titres of virus-neutralizing antibodies were determined as described earlier (Wolfgram *et al.*, 1986). The titre was defined as the highest dilution giving 100% inhibition of the virus infectivity.

Immunization. A.CA/SnJ mice, 5–6 weeks old, were immunized with 100 μg of cardiac myosin in 50 mM sodium pyrophosphate in a 1 : 1 emulsion with complete Freund's adjuvant (Difco) in both sides of the inguinal region. Seven days later the animals were re-injected using the same protocol. Twenty-one days after the first injection, sera were obtained by retroorbital bleeding.

Coupling of myosin to Sepharose. Sepharose 4B (Pharmacia) was activated with CnBr using the method of Parikh, Narch & Cuatrecasas (1974). Myosin was coupled to CnBr-activated Sepharose as described by Alvarez *et al.* (1987). The ratio was 1 mg myosin per ml Sepharose; the coupling efficiency was between 70 and 80%.

Absorption of sera, affinity purification of antibodies. Sera were diluted 1 : 10 in PBS, pH 7·2, and then incubated with three vol of myosin-coupled Sepharose with continuous shaking for 1 h at room temperature. The Sepharose beads were centrifuged at 100 *g* for 2 min and the supernatant representing absorbed serum harvested. Unbound protein associated with the Sepharose beads was removed by washing extensively with PBS. The beads were then rinsed with cold distilled H_2O and incubated with an equal volume of ice-cold glycine-HCl buffer, pH 2·8, for 15 min under continuous agitation. After centrifugation the supernatant containing the purified myosin antibodies was dialysed against cold PBS for several hours.

RESULTS

In previous studies (Alvarez *et al.*, 1987; Neu *et al.*, 1987) it was found that heart autoantibodies in mice with CB_3-induced myocarditis are directed against cardiac myosin. To determine whether

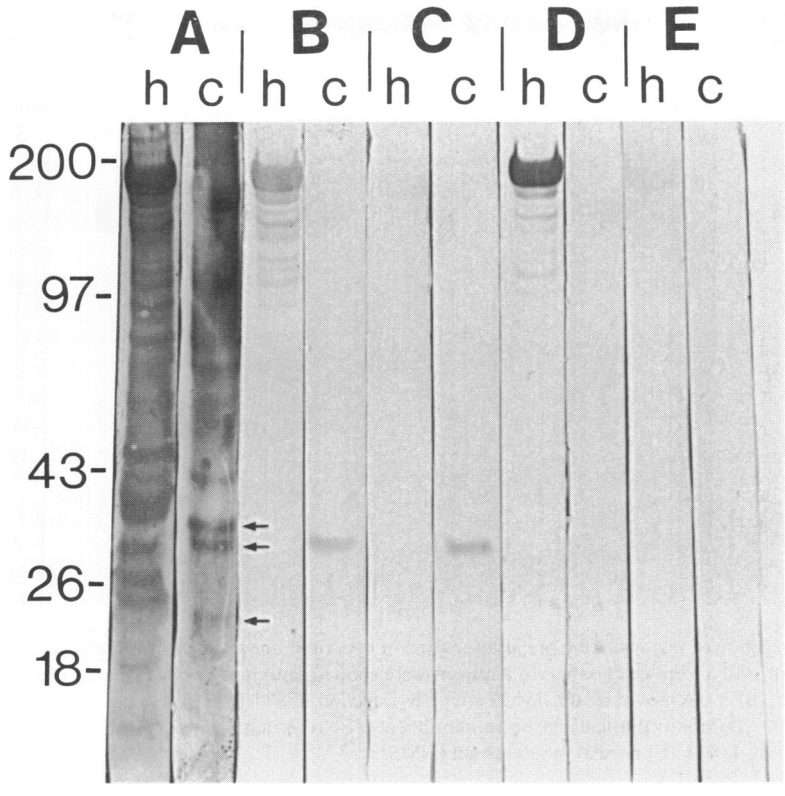

Fig. 1. Immunoblots showing the reaction of post-infectious serum against whole heart extracts (h) and CB_3 (c) preparations. The typical results from one out of three experiments (with three different serum pools) are shown. (A) Bio-Rad protein stain on nitrocellulose; arrows (from above) indicate the positions of VP1, VP2, and VP3, respectively; (B) A.CA mouse serum pool, 21 days after infection, dilution 1:200; (C) A.CA mouse serum pool, absorbed with cardiac myosin, dilution 1:100; (D) anti-myosin antibodies, affinity purified from the post-infectious A.CA serum pool; (E) normal mouse serum (NMS).

these autoantibodies are induced directly by the virus, we tested them for cross-reactivity with CB_3 antigens.

In the first experiment, a crude heart extract and a CB_3 concentrate were separated by SDS-PAGE and electroblotted onto nitrocellulose paper. The representative protein stain (Fig. 1A, h) shows numerous bands in the crude heart extract; the most prominent one is a 200 kD band, which has previously been identified as myosin heavy chain (Alvarez *et al.*, 1987). The bands found in the CB_3 preparation (Fig. 1A, c) display the typical pattern described by others (Beatrice *et al.*, 1980; Katze & Crowell, 1980) for the CB_3 capsid proteins. The pair of bands with a relative molecular weight (mol.wt) of 30 kD represents the capsid proteins designated as VP 1 and VP 2, whereas the band of about 20 kD is VP 3. All other bands are most probably Vero cell lysate proteins. Because of the lack of serum reactivity with uninfected Vero cell antigens (Fig. 2A), extensive purification of the virus was deemed unnecessary.

The Western blotted heart and virus preparations were then tested for their reactivity with three serum pools obtained from A.CA mice with CB_3-induced myocarditis. The major reaction (Fig. 1B) was with a 200 kD band found in the heart extract. In the CB_3 preparation, the serum recognized one band of the VP 1 VP 2 pair having a relative mol.wt between 28 and 30 kD. This band was tentatively identified as the capsid protein VP 2.

To test further if the anti-myosin in post-infection sera cross-reacted with the detectable CB_3 proteins, the serum pools were absorbed with cardiac myosin-coated Sepharose beads and tested

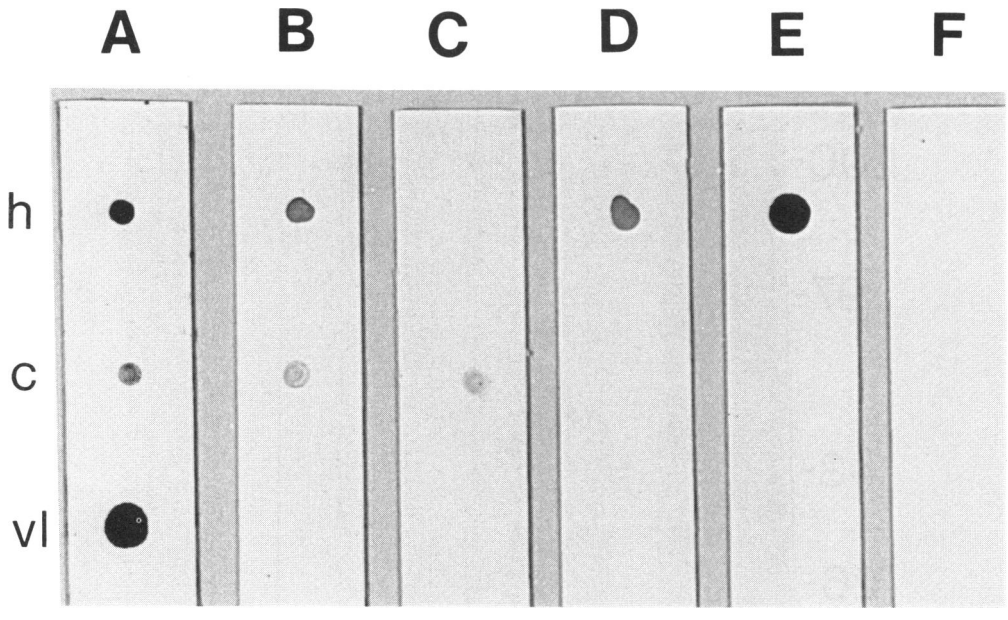

Fig. 2. The reaction of various serum preparations and of hyperimmune serum against native heart myosin (h), native CB_3 (c) and a Vero cell lysate (vl). Antigens were spotted onto nitrocellulose paper. (A) representative protein stain; (B) A.CA mouse serum, day 21 after CB_3 infection, 1:800; (C) same serum absorbed with cardiac myosin, 1:400; (D) affinity purified myosin autoantibodies; (E) hyperimmune serum from mice immunized with cardiac myosin, 1:800; (F) normal mouse serum (NMS).

against the two antigen preparations by Western immunoblotting. Cardiac myosin removed all the heart reactivity, confirming previous results (Alvarez *et al.*, 1987). However, the reactivity with CB_3 was unaffected (Fig. 2C). Conversely, the affinity purified anti-myosin antibodies reacted with the 200 kD protein in the heart extract, but were negative with the CB_3 preparation (Fig. 1D). These findings suggest that cardiac myosin autoantibodies found in mice with CB_3-induced autoimmune myocarditis do not cross-react with CB_3 antigens detectable by Western immunoblotting.

In order to separate the viral capsid proteins the SDS-PAGE was performed under reducing conditions, and therefore it is entirely possible that putative cross-reacting epitopes were denatured. For this reason we tested the serum preparations in an immunoblot using native test antigens. In addition, a pool of hyperimmune sera from A.CA mice that were immunized with cardiac myosin was analysed. Cardiac myosin, 1 μg, and 1 μl of the native virus suspension, were applied onto nitrocellulose paper and tested against the sera. To ensure that the serum reactivity was due to specific virus-antibody binding, a lysate of uninfected Vero cells was used as control antigen. This control was necessary because the virus was enriched, but not completely purified. The results (Fig. 2) show that the A.CA serum pools react with both native CB_3 and cardiac myosin. No reactivity was observed with the Vero cell lysate. The myosin-absorbed sera still reacted with CB_3, whereas the affinity purified myosin antibodies reacted with myosin only. The hyperimmune serum pool from the myosin immunized mice showed a strong reactivity with the myosin, but was negative with the virus.

To rank the antibody reactivities, the titres of the various serum preparations were determined using the dot blot assay system. The titres were expressed as the last serum dilutions giving a clearly visible stain. The results (Table 1) show that the postinfectious serum pool has a high titre against cardiac myosin and the CB_3 preparation (3200 and 6400, respectively). The myosin-absorbed serum had very little remaining activity against myosin (200); this is comparable to the anti-myosin activity in NMS. Repeated incubation with myosin-Sepharose resulted in non-specific antibody adsorption (not shown). The anti-CB_3 titre was almost as high as before absorption (3200). The affinity purified

Table 1. The reaction of serum preparations with native CB_3 suspension and cardiac myosin in the immuno-dot-blot test

Treatment of mice	Serum fraction	Test antigen	Titre
CB_3 infection*	Whole serum	Cardiac myosin	3200
		CB_3	6400
	Myosin-absorbed	Cardiac myosin	200
		CB_3	3200
	Anti-myosin†	Cardiac myosin	960
		CB_3	< 30
Myosin immunization‡	Whole serum	Cardiac myosin	25,000
		CB_3	400
None	Normal mouse serum (NMS)	Cardiac myosin	200
		CB_3	200

* Serum pool was obtained from A.CA mice 21 days after CB_3 infection.

† Affinity purified myosin autoantibodies.

‡ Hyperimmune serum pool from A.CA mice immunized with cardiac myosin (see Materials and Methods).

anti-myosin preparation had no detectable activity against the virus, whereas the titre against cardiac myosin was 960. The highest titre against cardiac myosin was found in the hyperimmune sera (25,000), but the reactivity with CB_3 was not significantly above that of NMS. Although the anti-myosin titre found in NMS was low, an antibody activity was still detectable (200). This finding is consistent with our previous findings (Neu *et al.*, 1987) and those of others (Dighiero *et al.*, 1983), suggesting that normal mice have a relatively high level of 'natural' anti-myosin antibodies. The results from these experiments confirm the data shown in Fig. 1, suggesting that the sera of CB_3 infected A.CA mice do not recognize epitopes which are shared by cardiac myosin and the virus. Moreover, immunization with cardiac myosin does not induce anti-CB_3 antibodies.

To determine if the myosin autoantibodies have any virus-neutralizing ability, the serum preparations were tested in a CB_3-neutralizing assay. It can be seen (Table 2) that the postinfectious A.CA serum pool had a neutralizing titre of 3200, and absorption with myosin did not affect this titre significantly. On the other hand, the eluted anti-myosin fraction had no virus-neutralizing capability. The hyperimmune sera from the myosin-immunized animals as well as NMS were also negative. Thus, the myosin autoantibodies found in CB_3 induced autoimmune myocarditis do not neutralize the virus, and immunization with cardiac myosin does not evoke neutralizing antibodies.

DISCUSSION

In the present study we investigated the possible role of CB_3 antigens in triggering autoantibody to

Table 2. Virus neutralizing titres of A.CA serum preparations

Treatment of mice	Serum fraction	Neutralizing titre
CB_3 infection*	Whole serum	3200
	Myosin-absorbed	1600
	Anti-myosin	< 30
Myosin-immunization	Whole serum	< 8
None	Normal mouse serum (NMS)	< 8

* See footnote to Table 1.

cardiac myosin in a murine model. CB_3 is a member of the picornaviruses and its structure has been well characterized. The capsid of the virus is not enveloped and is composed of four different polypeptides designated as VP 1, VP 2, VP 3, and VP 4 with approximate mol.wts of 29,000, 25,000, 21,000 and 4,500, respectively (Crowell & Philipson, 1971). In SDS-PAGE, VP 1 and VP 2 co-migrate, and the migration of VP 2 is anomalous compared to the mobilities of commonly used mol.wt standards. Therefore, the mol.wt of the capsid proteins originally had to be determined by co-electrophoresis with poliovirus capsid proteins (Crowell & Philipson, 1971; Philipson, Beatrice & Crowell, 1973). VP 4 can rarely be detected by SDS-PAGE (Beatrice *et al.* 1980; Katze & Crowell, 1980) and is weakly immunogenic or non-immunogenic (Talbot *et al.*, 1973). In Fig. 1 three bands with exactly the migration pattern described for VP 1–3 were found. Since the sera did not react with the Vero cell lysate, the bands could tentatively be identified as VP 1, VP 2 and VP 3. The virus antibodies from the CB_3 infected mice used in the present study seem to react exclusively with VP 2, the only capsid protein which can induce neutralizing antibodies (Beatrice *et al.*, 1980). It has been shown that not only VP 2, but also VP 1 and VP 3 are immunogenic in the rabbit if the animals are immunized with CB_3 emulsified in complete Freund's adjuvant (Beatrice *et al.*, 1980). But the mice used in our studies were inoculated with virus without using adjuvant in order to imitate natural infection, which might explain the apparent lack of an anti-VP 1 and anti-VP 3 reactivity.

The data presented in this communication provide evidence that autoantibodies to cardiac myosin, the major autoantigen in CB_3-induced autoimmune myocarditis, do not cross-react with or neutralize the virus. Additional evidence comes from the observation that the virus neutralizing antibodies appear on day 3 after the infection and persist for at least 7 weeks, whereas heart specific autoantibodies cannot be found before day 15 (Wolfgram, Beisel & Rose, 1985; Wolfgram *et al.*, 1986). Based on this difference in time of appearance, virus neutralizing antibodies and myosin autoantibodies are different populations.

We recognize that our data do not exclude the possibility that epitopes shared between cardiac myosin and Coxsackieviruses exist. Recently Saegusa *et al.* (1986) tested more than 60 monoclonal antibodies to Coxsackievirus B_4 and found that one of them was cross-reacting with myocardium. However, if there are epitopes shared between myosin and CB_3, they seem to be recognized neither by sera from CB_3-infected nor from myosin-immunized A.CA mice, as shown in the present study. On the other hand it is still possible that there are a few B cells which are capable of producing antibodies cross-reacting with CB_3 and cardiac myosin; this question could be explored by production of monoclonal antibodies to CB_3 and to cardiac myosin. We also cannot exclude the possibility that there are other autoantigens in addition to cardiac myosin. Finally, we have examined cross-reactivities only by autoantibodies and not at the level of T cells, which may well recognize different determinants.

Bearing these limitations in mind, our findings suggest that CB_3-induced autoantibody to cardiac myosin in A-strain mice cannot be attributed to an immune response that results from cross-reactivity between the virus and cardiac myosin. We propose that the production of autoantibody to cardiac myosin is secondary to the virus-mediated myocardial damage; CB_3 can be thought of as an indirect agent of this process. A few days after infection, the virus causes myocyte necrosis and inflammatory lesions. There is no evidence that these early lesions are associated with auto-immunity (Herskowitz *et al.*, 1985); they can be found regardless of whether or not the mice are susceptible to the late, autoimmune form of myocarditis. The myocyte necrosis, which is the hallmark of the early, viremic phase of myocarditis, leads to the exposure and perhaps alteration of myosin which, due to its poor solubility at physiological salt concentrations, persists in the area of necrotic cells (Haber, 1982). Therefore, we hypothesize that the autoimmune response that occurs in genetically predisposed strains may be stimulated by necrotic or damaged myocytes as myosin becomes accessible to the immune system.

The same mechanism which may cause autoimmunity in the A-strain mice is also thought to be responsible for the post-pericardiotomy and the myocardial infarction syndrome. These diseases are probably also caused by myocyte necrosis and the consequent release of myosin and/or other autoantigens, leading to an immune response to intracellular proteins (de Scheerder *et al.*, 1985). On the other hand, the pathological trigger which we are proposing for the CB_3-induced autoimmune myocarditis certainly is different from that suggested for other diseases associated with

postinfectious autoimmunity. For instance, in patients with rheumatic fever it has clearly been shown that heart autoantibodies are cross-reactive with streptococcal proteins (Zabriskie *et al.*, 1970; van de Rijn *et al.*, 1977). In addition, myosin and certain streptococcal strains share antigenic determinants (Krisher & Cunningham, 1985; Dale & Beachey, 1985).

We propose that the autoimmune form of viral myocarditis in the A *H-2* congenic strains of mice is initiated by an autoimmune response to cardiac myosin. If this hypothesis is correct, one has to predict that mice susceptible to this form of heart disease possess genes encoding a heightened immune response to cardiac myosin, which the non-susceptibles do not possess. In a forthcoming paper we demonstrate that this is indeed the case.

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