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Autoantibodies to cardiac myosin in mouse cytomegalovirus myocarditis

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SUMMARY

Myocarditis accompanies sublethal mouse cytomegalovirus (MCMV) infection in susceptible BALB/c mice and persists beyond the acute phase of infection, in the absence of demonstrable virus antigen but in the continuing presence of autoantibodies to cardiac muscle. Heart tissue autoantibodies of the IgG class were first detected by ELISA in sera at Days 3–5 post-infection (PI) and persisted to Day 100, in two strains of MCMV-infected mice which are susceptible (BALB/c) and resistant (C57BL/10) to MCMV-induced myocarditis. Analysis by immunoblot showed that autoantibodies in early immune sera (Day 10) from both mouse strains reacted with the contractile proteins troponin, tropomyosin and myosin, as well as with other unidentified polypeptides within normal mouse organ homogenates. However, the dominant reactivity of late immune sera (Day 100) was to a 200,000 molecular weight (MW) polypeptide in muscle homogenates identified as the heavy chain of myosin. Autoantibodies reacting with the cardiac or striated muscle isoforms of myosin were assessed by ELISA in BALB/c and C57BL/10 mice. At Days 28, 56 and 100 PI only the susceptible BALB/c strain had high titres of autoantibodies reacting with the cardiac isoform of myosin. Increasing the virus dose given to C57BL/10 mice resulted in slight increases in titres of anti-myosin antibody; however, the peak antibody titres did not approach those of BALB/c mice and persisting myocarditis did not develop. Absorption experiments showed that cardiac myosin-specific antibodies were present in immune sera from susceptible BALB/c mice at Day 100 but not in resistant C57BL/10 mice by ELISA and immunoblot. These results demonstrate that autoimmunity to myosin is a prominent feature of the humoral autoimmune response following MCMV infection, and that there are differences both in fine isoform specificity and titre of anti-myosin antibodies between strains of mice that develop persisting myocarditis and strains that do not. Cardiac myosin-specific autoantibodies may play an immunopathogenic role in CMV-induced myocarditis.

INTRODUCTION

Heart-reactive autoantibodies are a common finding in heart disease. Autoantibodies to heart proteins have been found in patients with viral myocarditis (Maisch *et al.*, 1982; Bolte & Schultheiss, 1978), idiopathic dilated cardiomyopathy (Schultheiss & Bolte, 1985), rheumatic carditis (Kaplan & Meyerserian, 1962), Chagas' disease (Cossio *et al.*, 1974), post-myocardial infarction syndrome (Kuch, 1973) and adriamycin cardiotoxicity (Maisch *et al.*, 1984). Immunoglobulin deposits in the myocardium have been reported in patients with congestive cardiomyopathy (Bolte & Schultheiss, 1978). Anti-heart autoantibodies found in the sera of patients with viral myocarditis react with sarcolemmal, myofibrillar and inter-fibrillary antigens (Maisch *et al.*, 1982) and may induce complement-mediated lysis of normal myocytes *in vitro* (Maisch *et al.*, 1983). In experimental models of coxsackievirus-induced viral myocarditis, heart-specific IgG autoantibodies are seen in Coxsackievirus B_3-infected mice (Neu *et al.*, 1987a) and heart antibody-producing B cells are seen in the myocardial infiltrate (Rose *et al.*, 1988). In particular, autoantibodies directed against the cardiac isoform of myosin are found in strains of mice susceptible to immunopathic myocarditis (Alvarez *et al.*, 1987; Neu *et al.*, 1987a) and can induce myocyte injury (Huber & Lodge, 1986).

In our murine model of cytomegalovirus (CMV)-induced myocarditis we have found that genetically defined inbred strains of mice developed varying disease patterns (Lawson *et al.*, 1989a). Virus inclusions were seen in myocytes at Days 3–10 post-infection (PI) only, accompanied by a predominantly

Abbreviations: ATP, adenosine triphosphate; B10, C57BL/10; BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; H&E, haematoxylin and eosin; i.p., intraperitoneal; LD_{50}, minimum virus dose lethal for 50% of animals; MCMV, mouse cytomegalovirus; MOBS, mouse osmolarity-buffered saline; MW, molecular weight; PFU, plaque-forming unit; PI, post-infection.

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mononuclear cell infiltrate, which persisted as late as Day 100 PI in some strains. Mouse strains with the BALB/c genetic background developed higher peak levels of myocarditis during the acute phase of infection than strains with the C57BL/10 (B10) background. Furthermore, mice with the susceptible BALB/c genetic background also developed persisting myocarditis, which occurred in the absence of viral antigens, as detected by immunoperoxidase histochemistry, and infectious virus as determined by plaque assay. In addition to the production of anti-viral antibodies of the IgM and IgG isotypes (Lawson, Grundy & Shellam, 1988), antibodies with specificities for normal tissues, including heart antigens, were induced by mouse CMV (MCMV) infection of mice (Bartholomaeus *et al.*, 1988). These autoantibodies were first detected in the sera at Day 7 PI by immunoperoxidase histochemistry and persisted as late as Day 100 PI. Myocarditis and the autoantibody response are dependent on functional T cells, since homozygous nude BALB/c mice do not develop the disease and do not produce autoantibodies (Lawson, O'Donoghue & Reed, 1989b). Analysis by Western immunoblot showed that autoantibodies reacted with a variety of polypeptides in 100,000 *g* supernatants of normal mouse cardiac muscle, striated muscle, liver, kidney and brain homogenates. In these earlier studies, reactivity with a 200,000 molecular weight (MW) polypeptide was consistently observed in brain and frequently seen in liver and kidney. However, the 200,000 MW band was not present in the electrophoresed preparations of striated muscle and heart, indicating that the myofibrils in these tissues were not solubilized in the preparation of homogenates and consequently were discarded with the pellets. In the present study we have used a different buffer to prepare normal organ homogenate preparations in which contractile proteins are represented. Myosin is a constituent of many tissues, although peptide mapping and amino acid sequence analyses have revealed the existence of closely related but distinct isoforms of myosin heavy chain (reviewed by Izumo, Nadal-Ginard & Mahdavi, 1986). In this study we have found that the predominant reactivity of late immune sera is to a 200,000 MW polypeptide, which was identified as the heavy chain of myosin. We show that significant strain differences occur in the production of heart-reactive autoantibodies both in titre and specificity. BALB/c mice, which are susceptible to the development of persisting myocarditis, also develop high levels of autoantibodies predominantly of the IgG isotype with specificity for the cardiac isoform of myosin.

MATERIALS AND METHODS

Mice

Specific pathogen-free inbred female BALB/c, BALB.K, BALB.B, BALB.G and B10 mice of 8 weeks of age were supplied by the Animal Resources Centre, Murdoch, Western Australia.

Virus

MCMV (Smith strain) was maintained by passage in weanling female BALB/c mice and stored in the gas phase of liquid nitrogen, as described elsewhere (Lawson, Grundy & Shellam, 1987). Normal, uninfected salivary gland homogenate served as a control.

Experimenal design

Mice were infected with 10^4 plaque-forming units (PFU) of MCMV (0·3 LD_{50} for BALB/c mice) by intraperitoneal (i.p.) injection. In another experiment, B10 mice were infected with 4×10^4 PFU of MCMV i.p., representing 0·3 LD_{50} for the B10 strain. On Days 0, 3, 5, 7, 10, 14, 28, 56 and 100 post-infection (PI), individual mice were bled, sera collected and stored at −20° until use and organs were removed and fixed in Bouin's Fluid as described previously (Bartholomaeus *et al.*, 1988).

Immunoperoxidase histochemistry

Immunoperoxidase histochemistry was carried out as described previously (Bartholomaeus *et al.*, 1988).

Myocarditis index

Bouin's Fluid-fixed heart sections stained with H&E were scored for myocarditis and given an index as described previously (Lawson *et al.*, 1989a).

Isolation of mouse myosin

Myosin was isolated from hearts of mice according to the method of Pollard (1982) with modifications. Hearts were removed from BALB/c mice and washed in ice-cold extraction buffer [0·5 M KCl, 0·1 M K_2HPO_4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 8 mM Na_2SO_3, pH 6·8]. The tissue was homogenized using a Waring blender (Thomas Co., PA) and Braun pestle homogenizer (FRG) and incubated on ice for 10 min with the addition of protease inhibitors [100 μl/10 ml buffer of leupeptin (Sigma, St Louis, MO), 1 mg/ml; trayslol (Bayer Leverkusen, FRG), 100,000 U/ml; pepstatin (Sigma), 1 mg/ml in DMSO; and phenylmethylsulphonyl fluoride (Sigma), 0·2 M in DMSO] followed by centrifugation at 10,000 *g* for 30 min at 4°. The pellet was re-extracted and three volumes of ice-cold 2 mM $MgCl_2$ were added to the pooled supernatants, the pH adjusted to 6·4 with 0·5 M acetic acid and the solution stirred for 15 min at 4°. The crude actomyosin precipitate was pelleted by centrifugation at 10,000 *g* for 20 min at 4° and dissolved in KCl-ATP buffer [1·0 M KCl, 0·02 M K_2HPO_4, 20 mM adenosine triphosphate (ATP), pH 7·0] using a Braun pestle homogenizer. Residual actin was pelleted by ultracentrifugation at 130,000 *g* for 1 hr at 4°. Actomyosin in the supernatant was preciptated overnight at 4° after stepwise (35–42%) ammonium sulphate fractionation using saturated ammonium sulphate (adjusted to pH 8·2 with 0·01 M EDTA, pH 7·0), pelleted by centrifugation and dissolved in cold KI-ATP buffer (0·6 M KI, 20 mM ATP, 5 mM DTT, 1 mM $MgCl_2$, 20 mM imidazole, pH 7·0). Myosin was separated from actin by chromatography. The solution was clarified by centrifugation at 10,000 *g*, 20 min at 4°, and applied to a column of A-15m resin (BioRad, Richmond, CA) behind a 50 ml zone of KI-ATP buffer. Myosin and actin were eluted with KCl column buffer (0·6 M KCl, 1 mM DTT, 10 mM imidazole, pH 7·0). Fractions containing myosin were precipitated by dialysis against an equal volume of saturated ammonium sulphate, dissolved in 50 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 8·5, at 1 mg/ml and stored at −20°. Skeletal muscle myosin was similarly prepared from the hindleg of mice except that lower concentrations of ATP (5 mM) were used, the preparation was not ultracentrifuged and was applied to a Sepharose 4B column (Pharmacia, Uppsala, Sweden). The purity of myosin preparations was checked by SDS–PAGE.

Preparation of tissue homogenate supernatants

Normal, uninfected BALB/c mice were killed by cervical dislocation. Striated muscle, cardiac muscle, brain, liver, kidney and salivary glands were excised and washed in cold high ionic strength buffer (20 mM Tris-HCl, 0·6 M KCl, 2 mM ATP, 2 mM $MgCl_2$) with 1 mg/ml protease inhibitors added [leupeptin 1 mg/ml in phosphate-buffered saline (PBS); trayslol 100,000 U/ml; pepstatin 1 mg/ml in DMSO; phenylmethylsulphonylfluoride, 0·2 M in DMSO]. Forty per cent (v/v) homogenates were prepared using a Waring blender followed by a Braun pestle homogenizer, centrifuged at 100,000 *g* for 60 min at 4° and the supernatants stored at −70°.

Enzyme-linked immunosorbent assay (ELISA)

Myosin preparations were diluted in carbonate–bicarbonate buffer with 50 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 9·6, and 0·25 μg/100 μl added to polystyrene microtitre plates which were incubated overnight at 4° before use in the ELISA as described previously (Bartholomaeus *et al.*, 1988). An ELISA index was calculated as described previously (Bartholomaeus, O'Donoghue & Reed, 1984). Briefly, the absorbance of the test sample was divided by the mean absorbance plus two standard deviations (SD) from the normal mean value of 10 normal controls (uninfected mouse sera). An index $\geq 1{\cdot}10$ was considered to be a positive response.

Filtration ELISA for detection of antibodies to tissue homogenates

A modified ELISA to that described previously (Bartholomaeus *et al.*, 1988) was developed, using high protein/nucleic acid retaining nitrocellulose membrane filters to retain a representative array of antigens involved in the heterogeneous autoantibody response. Tissue homogenate supernatants were adjusted to 20 μg/ml and 200 μl/well was adsorbed onto pre-wet Millititre 0·45 μm HA 96-well filtration plates (Millipore, Bedford, MA) for 15 min at room temperature, and 5% bovine serum albumin (BSA) was used in the blocking solution.

Polyacrylamide gel electrophoresis and immunoblot

Tissue homogenate supernatants and protein solutions adjusted to 1 mg/ml were dialysed against sample buffer [10 mM Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulphate (SDS), 40 mM DTT, 10 M urea, pH 8], denatured for 10 min at 100° and applied to 10% polyacrylamide gels with a 3% polyacrylamide stacking gel. Myosin preparations were denatured in the above buffer without the addition of urea. Identical gels were run, one of which was stained with Coomassie blue. Polypeptides from the remaining gels were transferred to nitrocellulose and immunoblot performed as described previously (Bartholomaeus *et al.*, 1988). Standard proteins of defined MW ranging from 14,000 to 200,000 were included in each gel (Bio-Rad; BRL, Gaithersburg, MD). Purified contractile proteins from rabbit muscle, including actin, myosin, troponin and tropomyosin, were obtained commercially (Sigma).

Coupling of myosin to Sepharose beads

The coupling of myosin to beads was performed according to the method of Alvarez *et al.* (1987). Myosin preparations were added to CNBr-activated Sepharose 4B (Pharmacia) at a concentration of 1·5 mg myosin/ml of damp beads. Coupling was performed overnight with mixing end-over-end followed by washing with 50 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 8·5. Residual active groups on the beads were blocked with 1 M ethanolamine, pH 9·0, for 2 hr at room temperature, the beads washed in 50 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 8·5, and washed alternatively (three cycles) in 0·1 M acetate buffer with 0·5 M NaCl, pH 4, and 50 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 8·5. The coupled beads were finally washed in 0·6 M KCl and equilibrated in PBS with mouse osmolarity (MOBS; 330 mOsmol) and stored at 4°. BSA was similarly coupled to the beads.

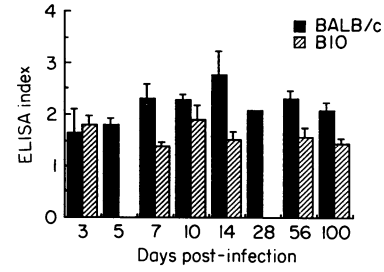

Figure 1. IgG autoantibody response to normal heart homogenate in BALB/c and B10 mice infected with 10^4 PFU of MCMV. The mean ELISA index ± SEM for eight mice is shown.

Absorption of sera

Undiluted sera were incubated with 5 volumes of myosin-coupled Sepharose or BSA-coupled Sepharose. After continuous mixing for 18 hr at room temperature, the beads were centrifuged at 100 *g* for 5 min and the absorbed sera saved. The beads were washed five times with MOBS and then once with cold double distilled water. Myosin-reactive antibodies were eluted from the beads by incubation for 15 min at 4° with one resin volume of 0·05 M acetate buffer, pH 2·8, with constant mixing. Following centrifugation at 100 *g* for 5 min, the eluted antibodies were dialysed overnight against MOBS at 4°.

Statistical analysis

Levels of significance were determined by the Wilcoxon rank sum test.

RESULTS

Antibody response to whole heart homogenate

Initially two strains of mice, the BALB/c strain, which develops severe levels of myocarditis persisting as late as Day 100 PI, and the B10 strain, which develops a mild, transient myocarditis (Lawson *et al.*, 1989a), were studied. Autoantibody to heart homogenates was assessed by filtration ELISA. Normal uninfected mice of both strains did not show serum reactivity to the whole heart homogenate preparation. However, antibodies were detected at Days 3–5 PI for both BALB/c and B10 virus-infected mice and persisted to Day 100 (Fig. 1). The peak titres of heart-reactive antibodies were seen at Day 10 (B10) and at Day 14 (BALB/c). Antibody titres in sera from BALB/c mice were significantly higher than those of B10 at Days 5 ($P < 0{\cdot}01$), 7 ($P \leqslant 0{\cdot}005$), 14 ($P \leqslant 0{\cdot}025$), 28 ($P < 0{\cdot}002$) 56 ($P \leqslant 0{\cdot}005$) and 100 PI ($P < 0{\cdot}005$). Heart antibodies of the IgM isotype from both BALB/c and B10 MCMV-infected mice were also demonstrated (data not shown).

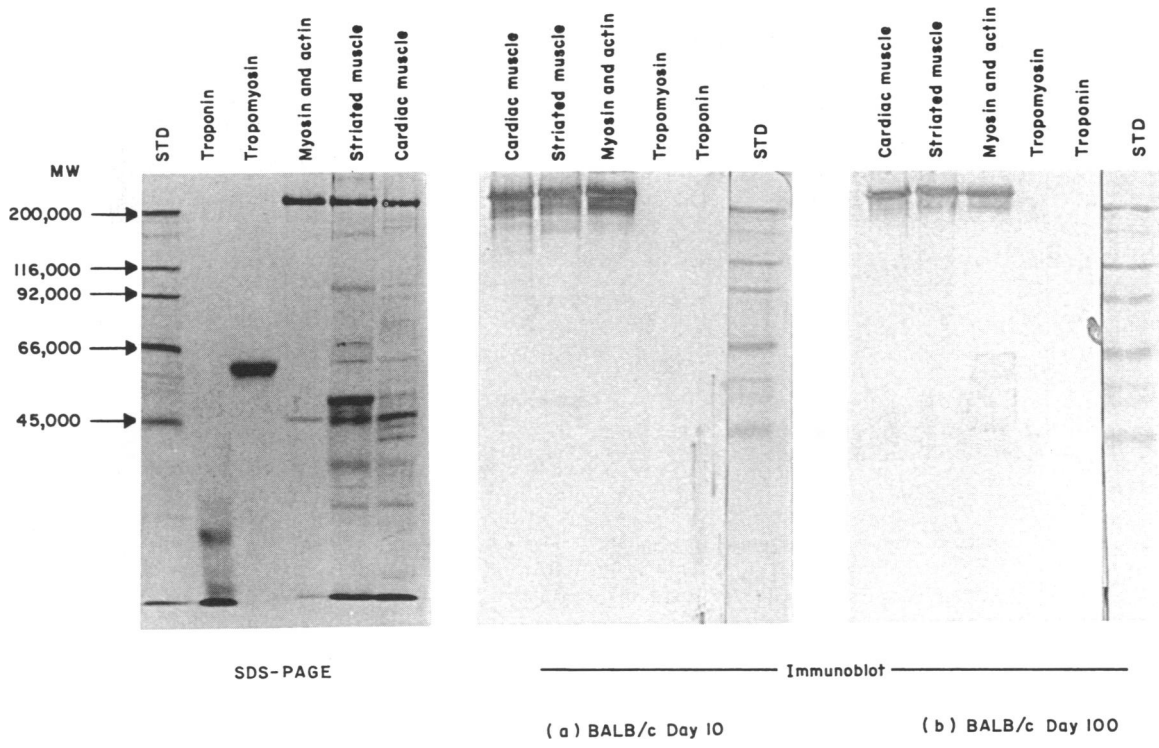

Figure 2. SDS–PAGE and immunoblot of 100,000 *g* supernatant proteins of normal mouse tissues and purified contractile proteins using serum from an individual BALB/c mouse 10 days (a) and 100 days PI (b) with MCMV. SDS–PAGE of MW standards and proteins is shown on the left and the MCMV-infected BALB/c mouse serum and alkaline phosphatase-labelled antisera to mouse immunoglobulins is shown on the right.

Polypeptide specificities of heart-reactive autoantibodies

The specificities of the heart-reactive autoantibodies were analysed further by SDS–PAGE and immunoblot using normal mouse organ homogenate preparations and commercially obtained preparations of purified contractile proteins. Normal BALB/c sera did not recognize any of the polypeptides represented in the organ homogenate preparations tested (kidney, salivary gland, liver, brain, striated muscle and heart). As previously reported (Bartholomaeus *et al.*, 1988), individual immune sera from MCMV-infected mice at Day 10 PI reacted with several polypeptides in each normal organ preparation. In both BALB/c (Fig. 2a, only muscle homogenates shown) and B10 sera at Day 10 PI, reactivity was consistantly observed against a polypeptide of approximately 200,000 MW in heart, striated muscle, salivary gland, liver, brain and kidney organ homogenates. In late immune sera (Day 100) from both BALB/c (Fig. 2b, only muscle homogenates shown) and B10 mice, several specificities were lost; however, reactivity with the 200,000 MW polypeptide remained prominent in every individual serum tested.

Early post-infectious sera (Day 10) from both BALB/c (Fig. 2a) and B10 virus-infected mice reacted with a number of purified heart contractile proteins, including troponin, tropomyosin and myosin, but not with actin. Only 1/9 sera of BALB/c mice (Day 10) recognized troponin, 6/9 recognized tropomyosin and 10/10 reacted with a 200,000 MW polypeptide, the heavy chain of myosin. A similar pattern of reactivity was seen in Day 10 sera of B10 mice, with 0/5 recognizing troponin, 2/4 reacting with tropomyosin and 4/4 reacting with myosin. Again, in late immune sera (Day 100) from both BALB/c (Fig. 2b) and B10 mice, reactivity to the 200,000 MW band of myosin remained while reactivity with tropomyosin, troponin or actin was no longer seen.

Antibody response to skeletal muscle myosin

To assess the reactivity of immune sera from MCMV-infected BALB/c and B10 mice to defined myosin isoforms, mouse skeletal muscle myosin was purified from the hindleg muscle and gave a single band of 200,000 MW by SDS–PAGE. Normal mouse sera diluted 1/80 did not have IgG or IgM antibody reactivity to skeletal muscle myosin in the ELISA. In addition, control sera taken at Days 10, 14, 57 and 113 post-inoculation of BALB/c mice with normal (virus-free) salivary gland homogenate did not have reactivity to skeletal muscle myosin (data not shown).

Antibody titres of the IgG isotype significantly higher than those from uninfected mice were observed at Days 10, 14 and 28 PI for BALB/c mice and at Days 7, 10 and 14 PI for B10 mice (Fig. 3a). Peak titres occurred at Day 10 PI for B10 and at Day 14 PI for BALB/c mice. Again, the BALB/c strain produced significantly higher autoantibody titres than the B10 strain at Days 14 ($P<0{\cdot}04$) and 28 PI ($P<0{\cdot}01$); however, titres to skeletal muscle myosin were no longer significantly elevated relative to normal sera in either strain at Days 56 and 100 PI. Antibodies of the IgM isotype reactive with skeletal muscle myosin were demonstrated in immune BALB/c sera at Days 7, 10 and 14 PI, with peak levels seen at Days 7–10 PI (Fig. 3b). Sera obtained from MCMV-infected B10 mice did not contain

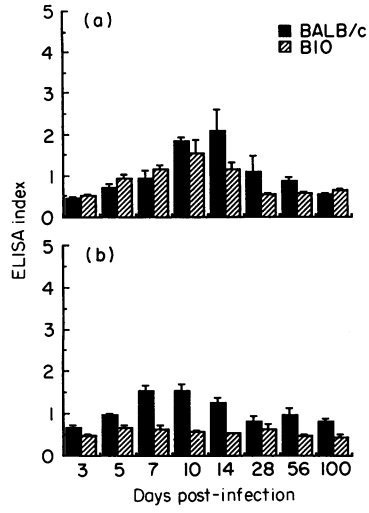

Figure 3. Autoantibody response to mouse skeletal muscle myosin in BALB/c and B10 mice infected with 10^4 PFU of MCMV. (a) IgG antibody responses and (b) IgM antibody responses are expressed as the mean ELISA index ± SEM for eight mice.

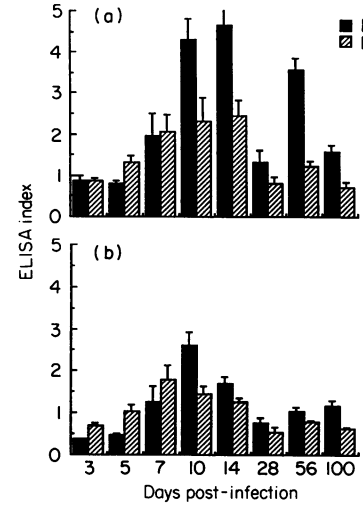

Figure 4. Autoantibody response to mouse cardiac muscle myosin in BALB/c and B10 mice infected with 10^4 PFU of MCMV. (a) IgG antibody responses and (b) IgM antibody responses are expressed as the mean ELISA index ± SEM for eight mice.

significantly positive levels of IgM autoantibodies at any of the time-points tested. No IgA anti-skeletal muscle myosin antibodies were found in immune sera (Day 10) of MCMV-infected BALB/c or B10 mice.

Antibody response to cardiac muscle myosin

Mouse cardiac myosin was purified and observed as a major band of approximately 200,000 MW by SDS-PAGE, with occasionally some intermediate MW polypeptides representing breakdown products of the heavy chain. Again, sera diluted 1/80 from uninfected mice did not react to cardiac myosin, and control sera taken at Days 10, 14, 56 and 113 post-inoculation of BALB/c mice with normal, uninfected salivary gland homogenate did not have reactivity to cardiac myosin by ELISA (data not shown).

Sera from both BALB/c and B10 mice infected with MCMV contained antibody to mouse cardiac myosin. Antibodies of the IgG isotype were first detected in sera taken at Day 5 PI of the B10 strain and at Day 7 PI of the BALB/c strain (Fig. 4a). Peak titres of this IgG antibody occurred at Day 14 for both strains and titres remained at significantly positive levels relative to that of normal uninfected mice at Day 100 in the BALB/c strain only. Sera from both strains of mice collected at Day 7 PI did not contain significantly different levels of antibody to cardiac myosin. However, the BALB/c strain produced anti-cardiac myosin IgG antibodies in titres significantly higher than those of B10 mice at Days 10 ($P < 0.02$), 14 ($P < 0.006$), 28 ($P < 0.002$), 56 ($P < 0.006$) and 100 PI ($P < 0.03$). The BALB/c strain produced IgG autoantibody to cardiac myosin in high titres (Fig. 4a) in comparison to those reactive with skeletal muscle myosin (Fig. 3a).

Antibodies of the IgM isotype reacting with the cardiac isoform of myosin were found in sera from both strains, appearing at Day 7 and remaining until Day 14 PI (Fig 4b). Peak titres occurred at Day 7 for B10 and at Day 10 PI for BALB/c mice. Again, sera from the BALB/c strain contained significantly higher titres of anti-cardiac myosin antibodies than those of B10 mice at Days 10 ($P < 0.01$) and 14 PI ($P < 0.02$). No IgA anti-myosin antibodies to the cardiac isoform were detected in immune sera (Day 10) of BALB/c or B10 mouse strains.

Increased virus dose in low-responder B10 mice

In order to investigate the influence of virus dose on myocarditis and the autoantibody response to myosin, B10 mice, which are approximately four times more resistant to lethal MCMV infection than BALB/c mice (Chalmer, Mackenzie & Stanley, 1977), were inoculated with 4×10^4 PFU of MCMV, equivalent to 0·3 LD_{50} for B10 mice, thus allowing a direct comparison of host humoral responses in B10 and BALB/c mice to an equivalent level of virus infection (0·3 LD_{50} for each strain). Similar levels of myocarditis were seen at Days 3, 5 and 7; however, at Day 10 PI, B10 mice had markedly lower levels of myocarditis than BALB/c mice (Table 1). B10 mice given the high virus dose did not develop persisting myocarditis, since inflammation of the myocardium was absent by Day 56 PI.

IgG anti-cardiac myosin antibody titres were increased at Days 5, 7 and 14 after inoculation of B10 mice with 4×10^4 PFU compared with B10 mice infected with 1×10^4 PFU of MCMV (Table 1 and Fig. 4a). However, IgM reactivity to cardiac myosin was not increased in B10 mice receiving 0·3 LD_{50} for the B10 strain (data not shown). Despite these slight increases in IgG autoantibody reactivity to cardiac myosin in B10 mice infected with 0·3 LD_{50} of MCMV, the peak levels obtained in B10 mice (2·80) did not surmount those produced in BALB/c mice (4·66) infected with 0·3 LD_{50}. Titres of both IgG and IgM antibody to skeletal muscle myosin were increased in B10 mice when the higher virus dose was used, to levels approximately equivalent to those of BALB/c mice (data not shown).

Absorption of sera with myosin isoforms

To determine the specificity of serum autoantibodies for myosin isoforms, immune sera from BALB/c and B10 mice taken at Days 10 and 100 PI were absorbed with cardiac or skeletal muscle myosin covalently coupled to Sepharose. Sera were also

Table 1. Comparison of myocarditis and anti-myosin antibody levels in B10 and BALB/c mice infected with strain-adjusted virus doses

	B10 (0·3 LD_{50})*		BALB/c (0·3 LD_{50})	
Day PI	Myocarditis†	Anti-myosin antibody‡	Myocarditis	Anti-myosin antibody
3	0·00±0·00	0·68	0·17±0·16	0·86
5	0·50±0·17	1·52	0·60±0·25	0·80
7	1·20±0·20	2·50	1·20±0·13	1·94
10	0·60±0·16	1·77	1·45±0·10	4·28
14	0·75±0·13	2·80	0·80±0·20	4·66
28	0·89±0·19	0·53	0·67±0·21	2·50
56	0·00±0·00	0·71	0·78±0·15	3·55

* Mice were infected with strain-adjusted 0·3 LD_{50} of MCMV. B10 mice received 4×10^4 PFU and BALB/c mice received 1×10^4 PFU of MCMV by the i.p. route.

† Myocarditis expressed as the mean myocarditis index (see text) of 5–15 mice per group ± SEM.

‡ ELISA index for IgG antibody reactivity to cardiac myosin.

Table 2. Anti-myosin antibody activity* after absorption or elution from various myosin isoforms

		OD				OD	
Sera		Cardiac	Skeletal	Sera		Cardiac	Skeletal
BALB/c Day 10 unabsorbed		1·86	1·88	B10 Day 10 unabsorbed		0·94	1·13
Cardiac	A	0·28	0·29	Cardiac	A	0·30	0·54
	E	0·76	0·66		E	1·42	0·88
Skeletal	A	0·36	0·33	Skeletal	A	0·36	0·43
	E	0·83	0·84		E	1·30	1·53
BSA	A	1·14	1·47	BSA	A	1·32	1·37
	E	0·23	0·17		E	0·11	0·17
BALB/c Day 100 unabsorbed		1·06	1·12	B10 Day 100 unabsorbed		1·22	1·29
Cardiac	A	0·24	0·34	Cardiac	A	0·32	0·26
	E	1·95	0·49		E	0·54	0·38
Skeletal	A	0·69	0·40	Skeletal	A	0·44	0·48
	E	1·48	1·70		E	0·78	0·78
BSA	A	0·82	0·99	BSA	A	0·72	0·84
	E	0·14	0·22		E	0·11	0·16

* An ELISA was used to determine the serum activity (IgM and IgG) after absorption or elution from the indicated myosin isoforms or BSA coupled to Sepharose. Day 10 sera were tested at a 1/20 dilution in the ELISA and Day 100 sera were tested at 1/10 dilution. Eluted samples were used at 1/5 dilution in the ELISA.
A, absorbed; E, eluted.

absorbed with BSA-coupled Sepharose as a control for non-specific absorption. The absorbed and eluted antibodies were analysed for specific activities against cardiac and skeletal muscle myosin by ELISA (Table 2) and immunoblot (Fig. 5). Absorption of sera with BSA-coupled Sepharose lowered the titre of anti-myosin antibody reactivity compared to that of unabsorbed sera (Table 2). No anti-myosin antibodies were eluted from the BSA-coupled Sepharose. Similarly, gelatin-coupled Sepharose and beads alone slightly reduced the intensity of the reaction of the absorbed sera with myosin, as observed by immunoblot, indicating some non-specific absorbtion.

Absorption of Day 10 BALB/c sera with cardiac myosin removed the anti-cardiac myosin reactivity as seen in the ELISA (Table 2). Similarly, absorption of this sera with skeletal muscle myosin completely abrogated reactivity to skeletal muscle myosin. Reactivity to skeletal muscle myosin was also removed after absorption of BALB/c sera (Day 10) with cardiac myosin and, conversely, absorption of sera with skeletal muscle myosin removed activity to cardiac myosin. These results were confirmed by immunoblot (data not shown). In contrast, although absorption of Day 100 BALB/c sera with skeletal muscle myosin completely abolished reactivity to skeletal muscle myosin, some reactivity to cardiac myosin remained (Table 2). This finding was reaffirmed using lower dilutions of serum in the ELISA; at 1/2 dilution the mean OD against cardiac myosin was 2·0, while the OD against skeletal muscle myosin was 0·4. This finding was also reproduced by immunoblot (Fig. 5a). Absorption of

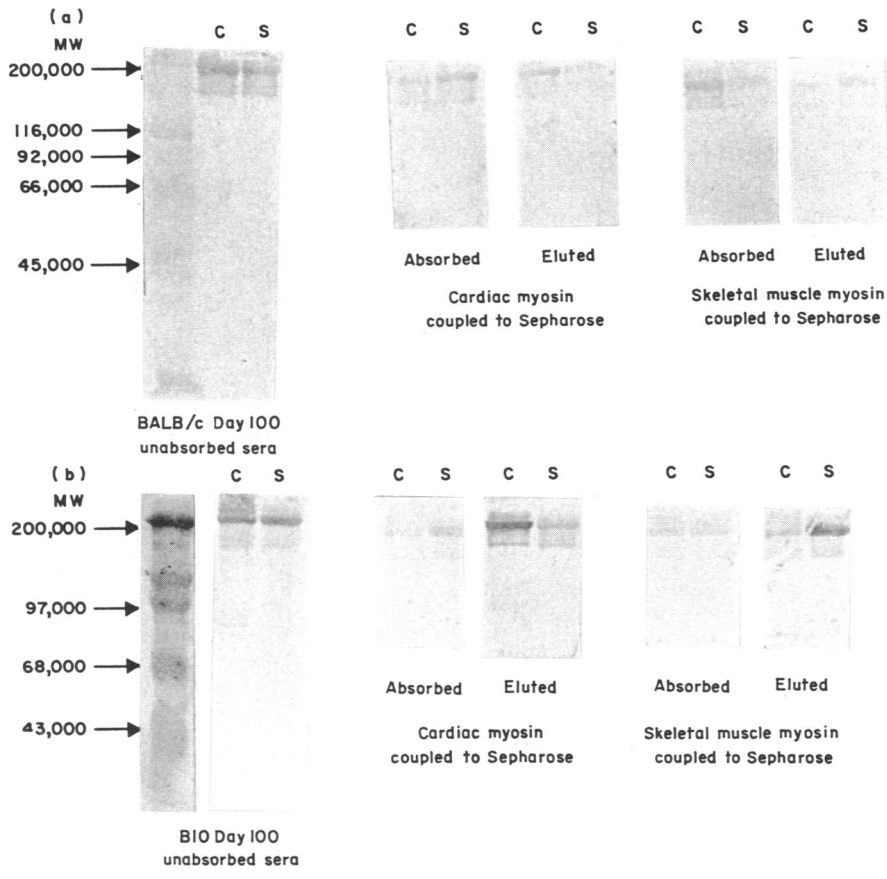

Figure 5. Immunoblot of purified normal mouse cardiac (C) and skeletal (S) muscle myosin using pooled sera from BALB/c mice 100 days PI (a) and from B10 mice 100 days PI (b) with MCMV. The sera was either unabsorbed (left), absorbed and eluted with cardiac myosin coupled to Sepharose (centre), or absorbed and eluted from skeletal muscle myosin coupled to Sepharose (right). SDS–PAGE of MW standards is shown on the far left.

BALB/c sera (Day 100) with cardiac myosin removed activity to both isoforms of myosin by ELISA. Although this result was reproducibly found by ELISA, by immunoblot some residual reactivity with skeletal muscle myosin was seen (Fig. 5a). These results suggest that in BALB/c sera taken at Day 100 after MCMV infection there are some antibodies specific for the cardiac isoform of myosin, and possibly some antibodies specific for the skeletal muscle isoform, in addition to a population of myosin autoantibodies that cross-reacts with both skeletal and cardiac muscle isoforms.

Absorption of B10 sera (Day 10 and Day 100 PI) with cardiac and skeletal muscle myosin abolished reactivity to both isoforms of myosin as determined by the ELISA (Table 2). However, by immunoblot, absorption of sera with the cardiac isoform did not completely remove activity to the skeletal isoform of myosin, whereas absorption of sera with skeletal myosin did effectively abolish reactivity to cardiac myosin (Fig. 5b, Day 100 only shown). Thus, there apear to be skeletal muscle myosin-specific antibodies and antibodies cross-reacting with both isoforms of myosin in B10 immune sera. No evidence of cardiac myosin-specific autoantibodies was obtained from the experiments with B10 mice. The observation of autoantibodies with specificity for striated muscle myosin, particularly in B10 mice, prompted examination for possible myositis. However, analysis by ELISA indicated that autoantibodies to striated muscle myosin were present in low titres, were not significantly elevated relative to normals after Day 28 PI, and preliminary histological assessment indicated that inflammatory infiltrates in striated muscle were not prominent in either BALB/c or B10 mouse strains.

DISCUSSION

Autoantibodies to heart contractile proteins contribute to the multiple autoantibody response following MCMV infection. Autoreactivity to a 200,000 MW polypeptide observed in salivary gland, brain, liver, kidney and muscle extracts may reflect cross-reactivity with different isoforms of myosin heavy chain. In the BALB/c strain, high titres of autoantibody to the cardiac isoform of myosin are produced in parallel with persisting myocarditis. Absorption studies showed that in this strain both heart-specific anti-myosin autoantibodies, as well as antibodies cross-reactive with skeletal muscle myosin, were induced. In contrast B10 mice produced low titres of autoantibody to cardiac myosin, and no cardiac myosin-specific autoantibodies were demonstrable.

Coxsackievirus B3 infection of mice also induces a post-infectious autoimmune myocarditis with cardiac myosin as the

predominant autoantigen (Neu *et al.*, 1987c). A two-stage pathogeneis of coxsackievirus B3-induced myocarditis is proposed: in the initial phase, focal myocarditis accompanies virus infection of the heart in all strains. In non-susceptible strains this is self limiting. However, strains with the appropriate immune response genes develop a second immunopathological phase, histologically distinct and characterized by the presence of heart-specific autoantibodies (Wolfgram *et al.*, 1986; Neu *et al.*, 1987b). These autoantibodies were shown to be reactive with the cardiac isoform of myosin (Neu *et al.*, 1987a). It is suggested that autoantibodies specific for cardiac myosin are a marker for immunopathological myocarditis (Neu *et al.*, 1987a).

There are interesting similarities between these murine models of coxsackievirus- and cytomegalovirus-induced myocarditis. In both models the development of myocarditis is influenced by both MHC and non-MHC genes (Lawson *et al.*, 1989a; Wolfgram *et al.*, 1986). As with coxsackievirus-induced myocarditis, we have found that the A/J strain is genetically susceptible to CMV-induced myocarditis and the C57BL/6 and B10 strains are resistant. In both models, the production of a population of autoantibodies with specificity for an epitope unique to the cardiac myosin molecule seems to be a key feature of the pathogenesis in genetically susceptible mouse strains.

However, in CMV-induced myocarditis, we have not observed a clear histological distinction between early and late myocarditis, as is reported for coxsackievirus-induced myocarditis (Rose *et al.*, 1987). Our observations suggest that there may be differences in the early pathogeneses of myocarditis induced by these dissimilar viruses. In coxsackievirus-induced myocarditis, present experimental evidence suggests that early myocarditis is due to virus-mediated damage of myocytes, with the consequent release of myosin to the extracellular spaces initiating the second 'immunopathic' phase in some strains (Neu *et al.*, 1987b). In MCMV-induced myocarditis, however, the initiation of myocarditis seems unlikely to be dependent upon virus infection of myoctes, since there is no correlation between severity of myocarditis and the number of infected cells in the heart (Lawson *et al.*, 1989a). For example, the BALB.K strain, in which little or no virus was seen in the heart, developed severe myocarditis, persisting until Day 100, in association with high titre anti-cardiac myosin autoantibodies. However, myocyte damage was clearly occurring, notwithstanding the apparent scarcity of virus-infected myocytes, and thus release of endogenous myosin may still be contributing to a process of autoimmunization in certain strains. The pathogenesis of MCMV-induced myocarditis is under further investigation in our laboratory.

The production of autoantibodies to myosin has been noted accompanying many infections, as well as following non-specific heart injury (De Scheerder *et al.*, 1985). 'Natural' monoclonal autoantibodies reacting mainly with myosin have been prepared from unimmunized BALB/c mice (Dighiero *et al.*, 1983, 1985). These monoclonals were found to have multiple reactivities, leading the authors to speculate that the determinant recognized my be a ubiquitous conformational structure, possibly associated with alpha-helical regions. The contribution of anti-myosin autoantibodies to the multiple organ reactivity seen in mouse sera following MCMV infection is yet to be defined. Natural self-reactive antibodies are mainly of the IgM class, of low affinity and are primarily cold-dependent (Rose, 1988). Both ourselves and other workers (Alvarez *et al.*, 1987) have observed some 'background' autoreactivity in normal mouse sera when reacted against purified murine myosins by the highly sensitive immunoblot technique. However, the switch to production of specific IgG antibodies to normal cardiac myosin in high titre may be a significant event in the pathogenesis of autoimmune myocarditis following MCMV infection in susceptible strains.

The mechanisms by which MCMV initiates autoimmunity and its role in the development of myocarditis are unclear. We have found that T cells are required for both the development of myocarditis and the autoantibody response following MCMV infection (Lawson *et al.*, 1989b). Mechanisms by which virus infection may trigger autoimmunity include virus-induced alteration of host cell membrane antigens, T-cell bypass, alterations in the MHC microenvironment, idiotype–anti-idiotype interactions, or 'molecular mimicry'. 'Molecular mimicry', or the sharing of antigenic determinants between infectious agents and self-tissue antigens, has been implicated in the pathogenesis of several autoimmune diseases (reviewed by Rose, 1988). We have recently found that a neutralizing monoclonal antibody to MCMV reacts strongly with myosin in heart, striated muscle, brain and liver extracts by immunoblot (C. M. Lawson, H. L. O'Donoghue, H. E. Farrell, G. R. Shellam and W. D. Reed, manuscript in preparation). This finding suggests that myosin and MCMV have shared epitopes.

CMV is endemic in human populations and most infections of otherwise healthy adults are subclinical. Due to the difficulties in diagnosis and identification of the aetiologic agent in viral myocarditis (Woodruff, 1980), the actual incidence of CMV-induced myocarditis is difficult to estimate. However, our mouse model has shown that asymptomatic MCMV infection can induce myocarditis and that in genetically susceptible individuals the myocarditis may persist for an extended period in the absence of detectable continuing viral stimulus. Here we report our findings of persisting elevated titres of cardiac myosin autoantibodies in susceptible mouse strains, and evidence of differences in fine specificity as well as titre between strains which develop the persisting disease and strains which do not. Humoral autoimmunity in human CMV myocarditis remains to be evaluated. Maisch *et al.* (1982) have reported that sera from 15 patients with CMV myocarditis showed a distinctive pattern of interfibrillary reactivity by indirect immunofluorescence; however, antibody to myosin was not found in these patients. Further studies in progress in our laboratory will clarify the significance of cardiac-myosin autoantibody in CMV-induced myocarditis.

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