Autoimmune Myocarditis Induced in Mice by Cardiac C-Protein

Cloning of Complementary DNA Encoding Murine Cardiac C-Protein and Partial Characterization of the Antigenic Peptides

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Abstract

Autoimmune myocarditis is considered to play a major role in the pathogenesis of dilated cardiomyopathy. A new autoimmune myocarditis model was attained by repeated immunization using murine cardiac C-protein with the immunological adjuvant, *Klebsiella pneumoniae* O3 lipopolysaccharide. For further analysis of a pathological epitope, the cDNA encoding C-protein was isolated; a fusion protein encoded by part of this cDNA induced myocarditis in SMA mice as well as in three other strains: DBA/1J (H-2^q), O20/A (H-2^{pz1}), and SJL (H-2^s). The nucleotide sequence and its deduced amino acid analysis revealed that this protein had immunoglobulin-like and fibronectin-like repeats. This study provides a new animal model of autoimmune myocarditis which may shed light on the pathogenesis of dilated cardiomyopathy. (*J. Clin. Invest.* 1994. 94:1026–1036.) Key words: autoimmune myocarditis • immunoglobulin superfamily • C-protein • dilated cardiomyopathy • lipopolysaccharide

Introduction

Dilated cardiomyopathy (DCM)[1] is a progressive disease characterized by a dilated heart with impaired systolic function. Since the pathogenesis remains obscure, it is usually diagnosed by ruling out valvular or coronary heart disease, excessive alcohol intake, high blood pressure, and systemic disorders that exhibit accompanying heart impairment, such as diabetes (1–3).

Infective myocarditis, in particular myocarditis associated with Coxsackie virus B3 (CB3), metabolic disease, or microvascular spasm is implicated in the pathogenesis of DCM (3, 4). However, several lines of evidence suggest that DCM is preceded by autoimmune myocarditis; autoantibodies against various cardiac proteins (5–8), abnormal expression of class II HLA-DR antigens on the myocardium, and a familial background support this notion (9–11). Although there is clear evidence that the autoantibodies of DCM patients react to several different proteins, the epitopes for experimental autoimmune myocarditis models and DCM still remain limited. Therefore, the cardiac muscle protein responsible for autoimmune myocarditis causing DCM must be identified.

Autoimmune myocarditis has been experimentally induced by cardiac myosin (12), a mixture of membranous proteins (13), and crude heart extract (14, 15). As a tissue-specific experimental autoimmune model (16–23), we reported myocarditis induced by syngeneic heart extract together with *Klebsiella pneumoniae* O3 lipopolysaccharide (KO3 LPS), an immunological adjuvant, in SMA mice (24). Here, we demonstrated that C-protein is the primary antigen which induces myocarditis. In addition, we cloned the cDNA encoding C-protein and identified part of the location of the antigenic epitope in various mice strains including SMA, DBA/1J, SJL, and O20/A.

Methods

Mice

SMA mice (H-2 K^s, D^{nondefined}) were maintained at the Institute for Laboratory Animal Research, Nagoya University School of Medicine. The H-2D haplotype has not yet been confirmed; it does not react to H-2D^{b,d,k,q,s}. The following were purchased from Seiwa Experimental Animals, Ltd. (Fukuoka, Japan) or bred in our Institute for Laboratory Animal Research: DBA/1J (H-2^q), DBA/2J (H-2^d), BALB/c (H-2^d), C57BL/6 (H-2^b), O20/A (H-2^{pz1}), SJL (H-2^s), A/J (H-2^a), AKR/J (H-2^k), and C3H/He (H-2^k).

Antigen preparation

Heart extract. The mouse heart was resected under deep anesthesia, frozen, and kept at −80°C until antigen preparation. All subsequent procedures were carried out at 4°C. The heart was rinsed and soaked in 0.01 M PBS, at pH 7.2, homogenized in 10 vol of PBS, and centrifuged at 700 *g* for 5 min. The supernatant was kept at −80°C until injection.

150-kD–rich fraction and 150-kD protein. High-salt extract of mouse heart was prepared according to the method of Neu et al. (25) with some modifications. The extract was passed through a Sepharose 4B gel filtration column (Pharmacia AB, Uppsala, Sweden) rather than Biogel A-15 m resin (Bio-Rad Laboratories, Richmond, CA). The fractions containing the 150-kD protein were collected, dialyzed against $^1/_{10}$ PBS, and then freeze-dried and resuspended in water. To purify the

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1. *Abbreviations used in this paper:* CB3, Coxsackie virus B3; DCM, dilated cardiomyopathy; Fn, fibronectin; KO3 LPS, *Klebsiella pneumoniae* O3 lipopolysaccharide; MBP, maltose binding protein; TCR, T cell receptor.

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*Table I. Induction of Murine Autoimmune Myocarditis in SMA Mice Using Different Antigens**

Experiment	Immunogen	Antigen dose × times	Induction of myocarditis
1	Heart extract (10%) + KO3 LPS	100 μl × 5	53% (10/19)
2	150-kD-rich fraction + KO3 LPS	100 μg × 3	88% (7/8)
3	Electroeluted 150-kD protein + KO3 LPS	50 μg × 3	100% (7/7)
		10 μg × 3	0% (0/3)
		2 μg × 3	0% (0/2)
	Electroeluted 150-kD protein	50 μg × 3	0% (0/2)
4	Electroeluted skeletal C-protein + KO3 LPS	50 μg × 6	0% (0/3)
5	Native C-protein + KO3 LPS	100 μg × 3	83% (5/6)
6	PBS + KO3 LPS	100 μl × 8	0% (0/20)

* Antigens mixed with 100 μg KO3 LPS or without LPS were injected at 4-wk intervals.

150-kD protein, this fraction was separated with 7.5% SDS-polyacrylamide gel (5 mm thick, 13 cm wide, and 9 cm high). After staining with Coomassie blue, the 150-kD bands were cut from the gel and eluted electrophoretically (model 422 electroeluter; Bio-Rad Laboratories). The eluted protein, precipitated by the addition of 10 vol of acetone, was then resuspended in water.

Native C-protein. C-protein was purified by the method of Hartzell and Glass (26) with some modifications. In place of a 2.5 × 35-cm hydroxyapatite column (high-resolution powder; Calbiochem-Novabiochem Corp., San Diego, CA) and a 1.6 × 35-cm DEAE-Sephacel column (Pharmacia AB), we used 1.5 × 3.8-cm HA-ultrogel (Sepracor S.A., Villeneuve la Garenne, France) and 1.5 × 3.8-cm Q Sepharose Fast Flow (Pharmacia AB). Skeletal muscle C-protein was prepared from murine sacral muscles by the same method and further purified electrophoretically.

Human cardiac C-protein. A human left ventricle was obtained at the Nagoya University Hospital from a 70-yr-old female who died of pancreatic cancer. The heart, which was resected at autopsy about 1 h after death, was histologically normal. The heart tissue was rinsed in PBS and kept at −80°C until sample preparation. C-protein was purified by the method described above.

KO3 LPS preparation

KO3 LPS as an adjuvant was prepared as described previously (27, 28) from the culture supernatant of a decapsulated mutant strain LEN-1 (O3:K1$^-$) derived from the *K. pneumoniae* Kasuya strain. In brief, the microorganisms were cultured in a synthetic medium at 37°C for 2 d. The organisms were killed by the addition of formalin and then concentrated 10-fold. After removal of bacterial cells by centrifugation, LPS in the supernatant was precipitated by adding ethanol and then treated three times with a chloroform/butanol mixture (1:5) for deproteinization.

Induction and assessment of myocarditis

6–8-wk-old mice were injected subcutaneously in the inguinal region with either 100 μl of the heart extract or PBS, 150-kD-rich fraction (100 μg), native C-protein (100 μg), 50 μg of electrophoretically purified C-protein, and fusion proteins (100 and 50 μg) together with KO3 LPS at 4-wk intervals. When mice developed clinical symptoms such as cyanosis and dyspnea, they were bled from the subaxillary artery under ether anesthesia and then killed by an overdose of anesthetic. All other mice were followed for 4 wk after the last immunization. For the diagnostic evaluation by the cardiac histology, all hearts were resected, fixed in 20% formalin/PBS, and stained by hematoxylin and eosin. Mice were considered negative for myocarditis when they had not developed myocarditis even after three repeated immunizations with the purified cardiac C-protein, or five times with the heart extract and fusion proteins, or six times with skeletal C-protein and PBS. Fusion protein P16-4 at 100 μg induced myocarditis in all SMA mice before the last immunization.

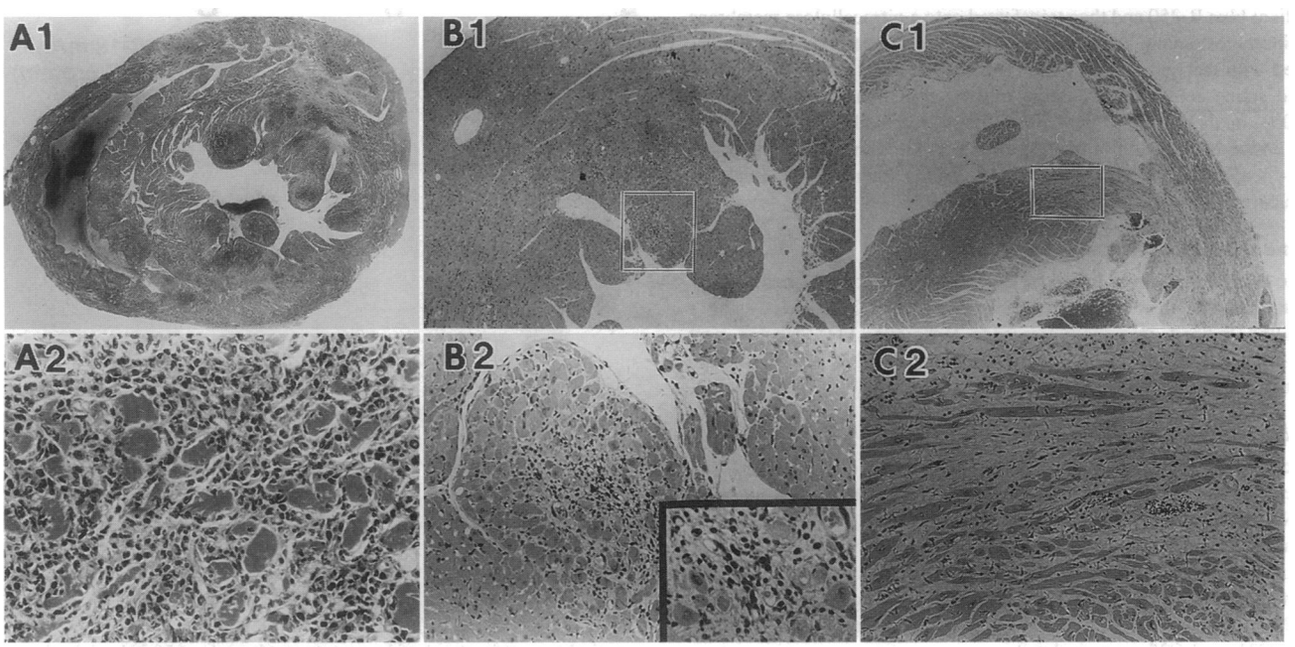

Figure 1. Autoimmune myocarditis induced by repeated injections of syngeneic heart extract mixed with KO3 LPS. Inflammatory responses were varied in individual mice from severe changes (*A1* and *A2*) to minimum changes (*B1* and *B2*) and chronic changes (*C1* and *C2*). Note the diffuse myocyte necrosis and inflammatory infiltrates (hematoxylin and eosin staining).

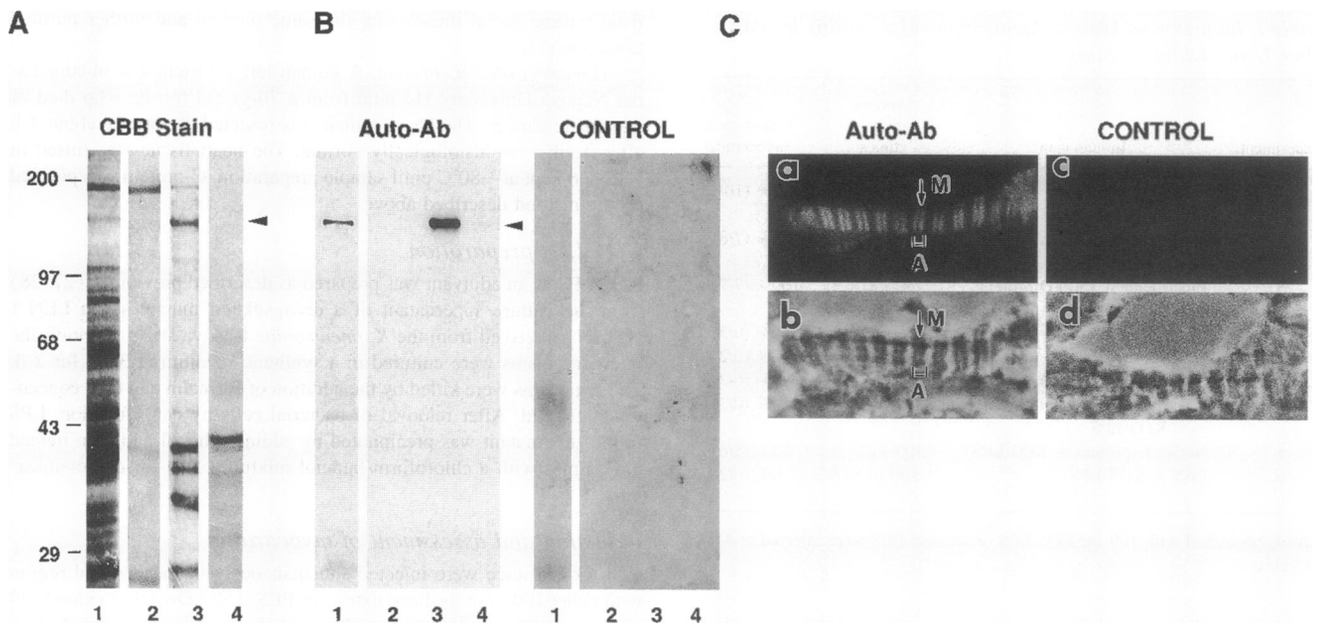

Figure 2. 150-kD protein is the dominant antigen recognized by the autoantibody from heart homogenate immunized mice. (*A*) SDS-polyacrylamide gel electrophoretic banding pattern of the heart extract and fractionation of high-salt extract of murine heart: lane *1*, heart extract; lane *2*, myosin (200 kD); lane *3*, 150-kD protein; and lane *4*, actin (43 kD). (*B*) Accompanying immunoblot with sera (Ab 1) obtained from immunized mice and preimmunization mice. The band at ~ 150 kD was detected preferentially by the autoantibody but not by the preimmunization sera (*CONTROL*). Note that preimmunization sera was diluted at 1:100, and autoantibody was diluted at 1:500. In addition, the nitrocellulose transfer was developed longer than that of autoantibody. (*C*) Immunofluorescent microscopic localization of the 150-kD protein in frozen sections of mouse heart (*a* and *c*) and phase-contrast image (*b* and *d*). The 150-kD protein was concentrated at the level of the A band with an unstained region in the center of each band (*a*). Preimmunization sera did not stain the cardiac cell (*c*).

Gel electrophoresis and immunoblotting

SDS-PAGE was performed according to the discontinuous Tris-glycine system of Laemmli (29). The slab gels were stained with Coomassie brilliant blue R-250 and then transferred onto a nitrocellulose membrane for immunostaining. Strips of the nitrocellulose membranes were incubated with test sera from immunized mice (diluted 1:500 in Tris-buffered saline containing 5% skim milk), preimmunized mice (diluted 1:100), mAb, and DCM patients or control sera (diluted 1:40) for 1 h. For antibody detection, a blotting detection kit with alkaline phosphatase–conjugated immunoglobulin (Amersham International, Buckinghamshire, UK) was used.

Immunohistochemistry

Sera, diluted 1:50, were tested on cryostat sections of the hearts (2-μm thick, 1% formaldehyde fixed, 0.2% Triton X-100 treated), using a standard indirect immunofluorescence technique. FITC-labeled goat anti–mouse Ig (Amersham International) was used at a 1:50 dilution. Sections were viewed on an ultraviolet microscope.

Isolation and sequencing of cDNAs

Mouse heart λgt11, oligo(dT)-primed expression cDNA library was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The initial cDNA clone, C26, was isolated using autoantibody of a myocarditis mouse according to the method described by Huynh et al. (30). The C26 fragment was then labeled by means of the DIG labeling kit (Boehringer Mannheim Corp., Indianapolis, IN) and used to screen the library according to the procedure developed by the manufacturer. Three clones, C16, C31, and C35, were isolated.

All clones were subcloned into pBluescript SK(−) and sequenced with 7-deaza Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH). To determine the sequence of long inserts, the

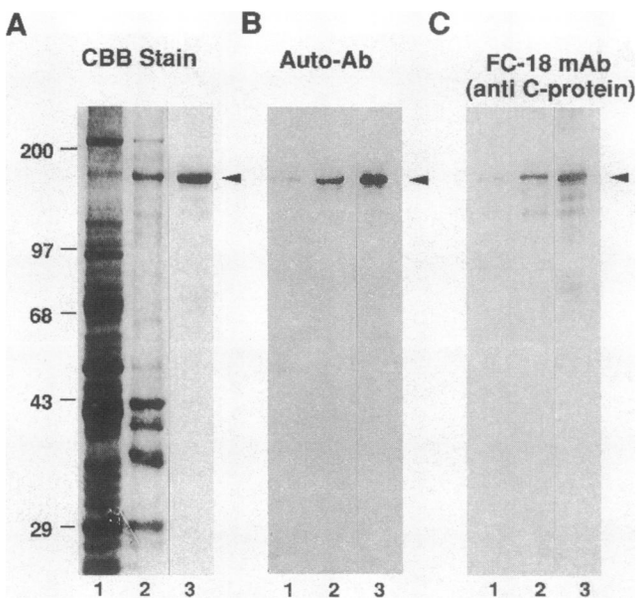

Figure 3. Identification of the 150-kD protein and C-protein. (*A*) Coomassie blue–stained gel of heart extract (*lane 1*), 150-kD–rich (*lane 2*), and purified C-protein (*lane 3*). Accompanying immunoblots with the autoantibody (Ab 1) (*B*) and with anti–C-protein mAb FC-18 (35) (*C*), which was donated by T. Obinata (Chiba University, Japan).

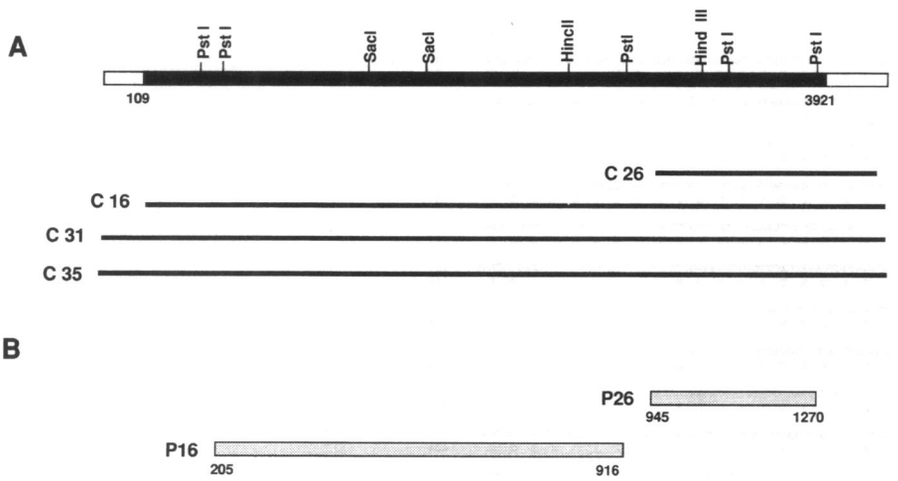

Figure 4. Mouse cardiac C-protein cDNAs and fusion protein fragments. (*A*) Restriction map and cDNA fragments. *Closed box,* the coding region; *open box,* the untranslated region. (*B*) Two fusion proteins (P16-4 and P26) are expressed in *E. coli* from Pst1 fragment of C16 (residue 205–916) and C26 (residue 945–1270).

plasmids were linearized and unidirectionally deleted using the Deletion Kit for Kilo-Sequence (Takara Shuzo Co., Ltd., Kyoto, Japan). The deletion mutants were also sequenced, as described above.

Production of fusion proteins in Escherichia coli

C26 and the PstI-PstI fragment of clone C16 were inserted in frame into the pMAL-CRI (New England Biolabs Inc., Beverly, MA). Fusion proteins and maltose binding protein (MBP) were expressed in XL1/Blue (RIKEN DNA Bank, Tsukuba, Japan) which was synthesized according to the procedure described by the manufacturer, and *E. coli* lysate was separated by SDS-PAGE and purified electrophoretically as mentioned above.

DCM patients

The DCM patients were 10 men and 6 women, aged 37–67 yr (mean±SD; 53.9±9.4 yr). They were diagnosed with DCM in accordance with the criteria described by Caforio et al. (8). Echocardiographic assessments showed that left ventricular end-diastolic and end-systolic echocardiographic dimensions were 54–74 mm (63.1±6.2) and 42–68 mm (54.0±8.4), respectively, and the ejection fraction was 15–54% (34.9±12.3). For controls, we examined 83 healthy volunteers, aged 20–54 yr (mean 41.0 yr).

Results

Autoantibody recognizes a 150-kD protein. Myocarditis was induced by the immunization of the combination of heart extract and KO3 LPS in 10 of 19 mice (Table I). This myocarditis was histologically characterized by inflammatory responses such as myocyte necrosis and lymphocyte infiltrates, from minimum (Fig. 1 *B*) to severe changes (Fig. 1 *A*) and chronic changes (Fig. 1 *C*). Sera (Ab 1), obtained from the myocarditis mice, showed a protein with a molecular mass of 150 kD on immunoblotting (Fig. 2 *B,* lane *1*). Based on the Coomassie stain of heart homogenate (Fig. 2 *A,* lane *1*), it was suspected that the 150-kD protein is one of the major constituent proteins in the cardiac cell. First, we passed the crude high-salt extract from the mouse heart through a gel filtration column and analyzed each fraction by immunoblotting with Ab 1. As Fig. 2 *A* shows, the 150-kD protein was concentrated in the fractions eluted after myosin (Fig. 2 *A,* lane *3*).

The fractions containing the 150-kD protein (100 μg) and the electrophoretically purified 150-kD protein (50 μg) induced myocarditis in almost all immunized mice (87.5 and 100%) (Table I). The incidence of myocarditis was dose dependent. When 10 and 2 μg of electroeluted 150-kD protein were used, no myocarditis was induced (Table I). Thus we suspect that at least 50 μg of C-protein is necessary for the induction of myocarditis. In control experiments, electrophoretically purified skeletal-type C-protein (50 μg) from murine sacralis muscle failed to induce either myocarditis or myositis (back and leg muscles were examined). PBS (100 μl) mixed with KO3-LPS did not induce myocarditis in SMA mice under the same experimental conditions (Table I).

Autoantibodies from the 150-kD protein–induced myocarditis mice showed immunofluorescent stained A bands with unstained region in the center of each band (Fig. 2 *C*).

The 150-kD protein is C-protein, and purified C-protein induces myocarditis. Due to the molecular mass (150 kD), the localization at the A bands, and the abundance in the heart, we were encouraged to examine whether the 150-kD protein and C-protein were identical. C-protein has been identified as one of the major constituents of thick filaments from skeletal muscle and shows a molecular mass of ~ 150 kD (31). The existence of cardiac-type C-protein has also been reported (32–34). Therefore, we performed immunoblotting analysis of the heart homogenate (Fig. 3 *A,* lane *1*) and the 150-kD–rich fractions (Fig. 3 *A,* lane 2) with anti–C-protein mAb, FC-18 (35). This mAb clearly recognized the 150-kD protein as well as Ab 1 (Fig. 3, *B* and *C*).

Then we purified C-protein from the murine heart in a native form (26), using the conventional column chromatography (Fig. 3 *A,* lane *3*). The purified native C-protein (100 μg) effectively induced myocarditis in SMA mice (83%, $n = 6$) when immunized together with KO3 LPS (Table I).

Isolation of cDNA encoding the cardiac C-protein. Using the autoantibody (Ab 1), we screened mouse heart λgt11 expression libraries to isolate cDNA fragments encoding the 150-kD protein (C-protein) and cloned one positive phage recombinant, C26 (Fig. 4). To obtain the full length of this protein, we then screened the same libraries with a probe from the 5′ end of C26. Through a series of rescreening experiments, four overlapping clones including C26 were isolated, and their restriction maps showed that they overlap with each other and constitute a single type of cDNA.

Fig. 5 shows the complete nucleotide and deduced amino

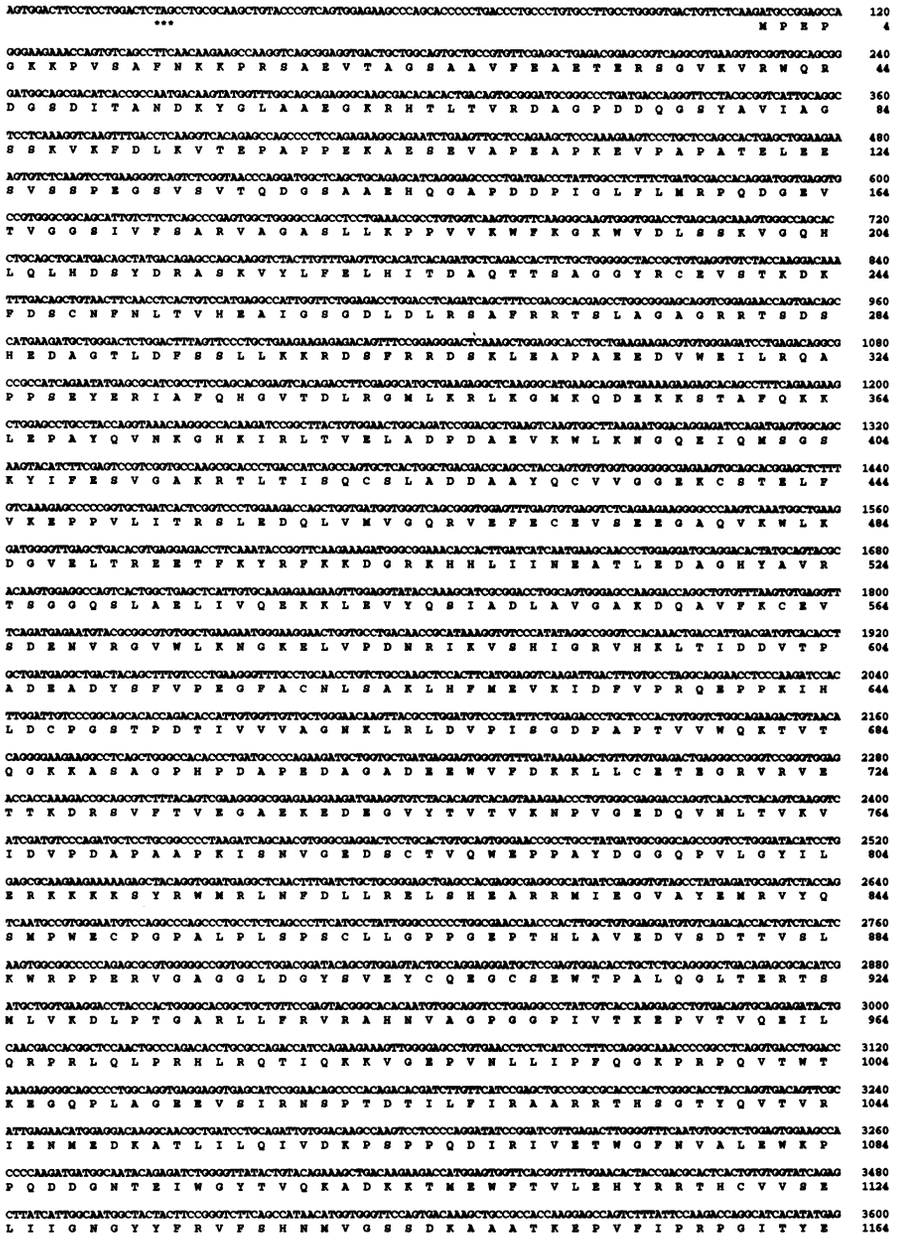

Figure 5. Nucleotide and deduced amino acid sequence of mouse heart C-protein. The stop codon TGA (residues 3919–3921) and the stop codon preceding the open reading frame (residues 22–24) are marked with asterisks. The polyadenylation consensus sequences found in the 3′ untranslated sequences are underlined.

acid sequences of the cloned molecule. In the 4224-nucleotide sequence, the longest open reading frame, with 3810 nucleotides, begins from the ATG codon at nucleotide 109 and ends with the TGA signal for termination at nucleotide 3919, thereby coding for a protein of 1,270 amino acids with the predicted mass of 140,621 daltons. The molecular mass of this protein on SDS-PAGE (150 kD), the high G + C content at positions 25–108 (64%), and one in-frame TAG stop codon (residue 22–24) precedes the ATG start codon (residue 109–111).

Comparison of the sequence of cardiac and skeletal C-protein. Recently, chicken (36, 37) and human skeletal C-protein (38) were cloned. Examination of the amino acid sequence of mouse cardiac C-protein revealed that this C-protein has 53% sequence identity with chicken fast isoform and 45% with human slow isoform by means of the amino acid homology program (GENETYX Software Development Co., Tokyo, Japan). The cardiac isoform (1,270 amino acids) is ~ 140 amino acids longer than two skeletal isoforms (1,132 amino acids in chicken

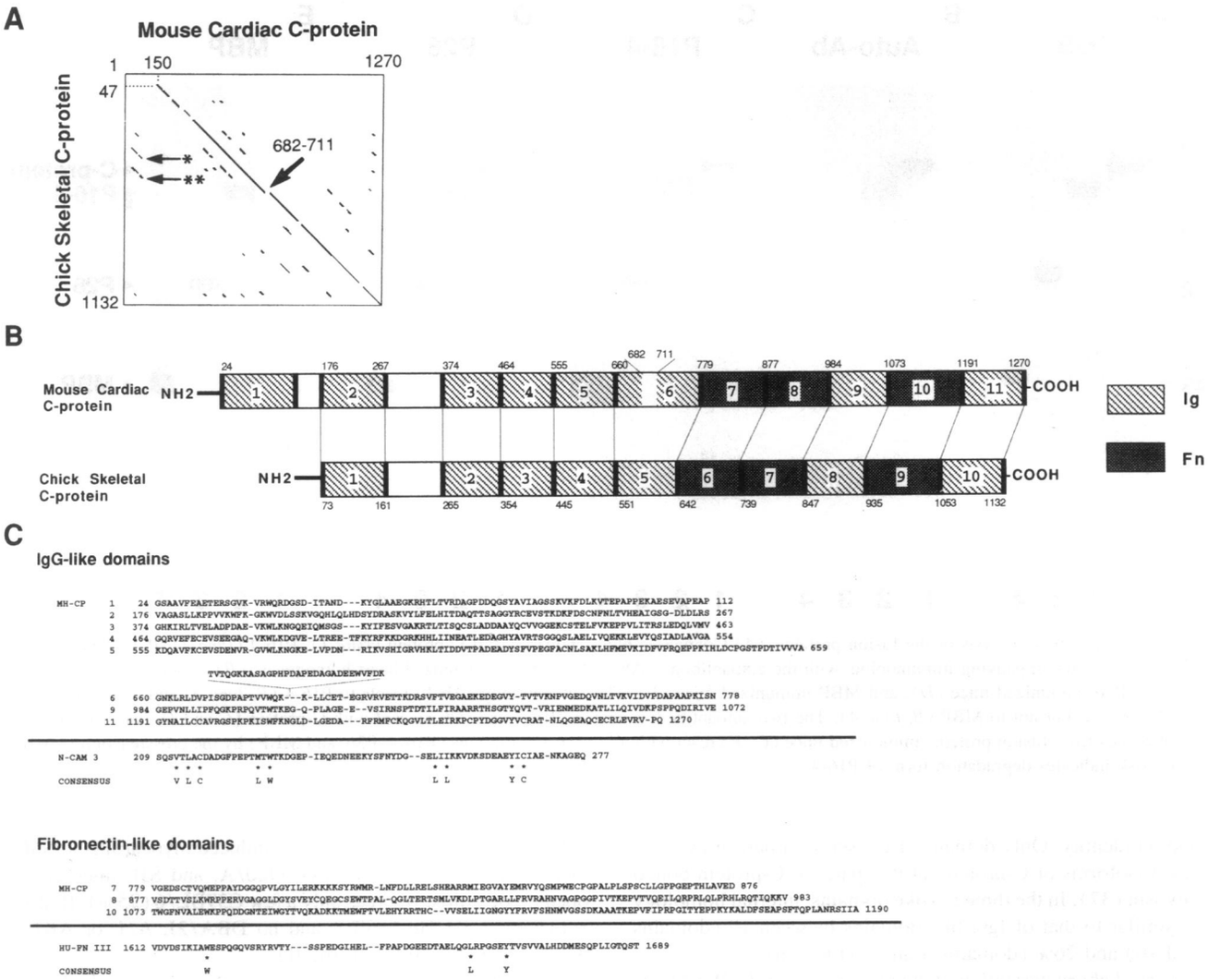

Figure 6. Analysis of the structure of mouse cardiac C-protein. (*A*) Diagonal dot matrix comparison between chick skeletal fast-isoform C-protein (37) and mouse cardiac C-protein was made using the GENETYX HARRPLOT program (unit size 22, and dot plot score 2.1) (39). Amino acid 150–1270 of cardiac isoform has a striking homology to amino acid 47–1132 of chicken skeletal isoform. The homology gap corresponding to the amino acid insertion (residue 682–711) in domain 6 of cardiac C-protein is indicated by the bold arrows. About 100 NH_2-terminal amino acids show strong homology to two IgG-like domains (domains 3 and 4) of skeletal C-protein (*small arrows,* * indicating domain 3, ** indicating domain 4). (*B*) Schematic structure of cardiac C-protein and chick skeletal C-protein consisting of two repeated motifs, IgG-like and Fn-like domains. Mouse cardiac C-protein domain 2–11 corresponded to chick skeletal isoform domain 1–10. (*C*) Alignment analysis of amino acid sequence of individual domain of murine heart C-protein (*MH-CP*). Numbers 1–11 on the left indicate positions in the C-protein deduced amino acid sequence. Alignment was done by inspection. IgG-like motifs of C-protein are compared with chicken neural cell adhesion molecule (*N-CAM 3*) (41) and a consensus for the immunoglobulin C-2 set domains (*CONSENSUS*) (40), Fn-like domains to human Fn type III repeat (*HU-FN III*) (42), and a consensus for the Fn type III domains (*CONSENSUS*) (43).

fast isoform [37], 1,138 in human slow isoform [38]). As shown in Fig. 6, *A* and *B,* amino acid 150–1270 of cardiac isoform had striking homology to amino acid 47–1132 of chicken skeletal isoform using the HARRPLOT program (GENETYX Software Development Co.) (39). However, there was a homology gap between 682 and 711 amino acids of cardiac isoform, indicating a 30-amino acid insertion in one of the IgG-like domains, repeat 6. About 100 NH_2-terminal amino acids showed strong homology to two IgG-like domains of skeletal isoform, domain 3 (amino acids 354–444) and domain 4 (amino acids 445–550). This additional immunoglobulin-like domain has four of nine IgG consensus sequences (40) and internal homology to cardiac isoforms domains 3, 4, and 5 with 26, 26, and 23% identity, respectively.

Fig. 6 *C* shows the alignment of amino acid sequence of individual domain. IgG-like motifs of C-protein are compared with chick neural cell adhesion molecule (41) and a consensus for the Ig C2 set domain (40). Fibronectin (Fn)-like domains are compared with human Fn type III repeats (42) and a consensus for the Fn type III domain (43). In the eight IgG-like domains shown in Fig. 6 *C,* internal homology was seen between 17 (domains 2 and 6, and 4 and 6) and 29% (domains

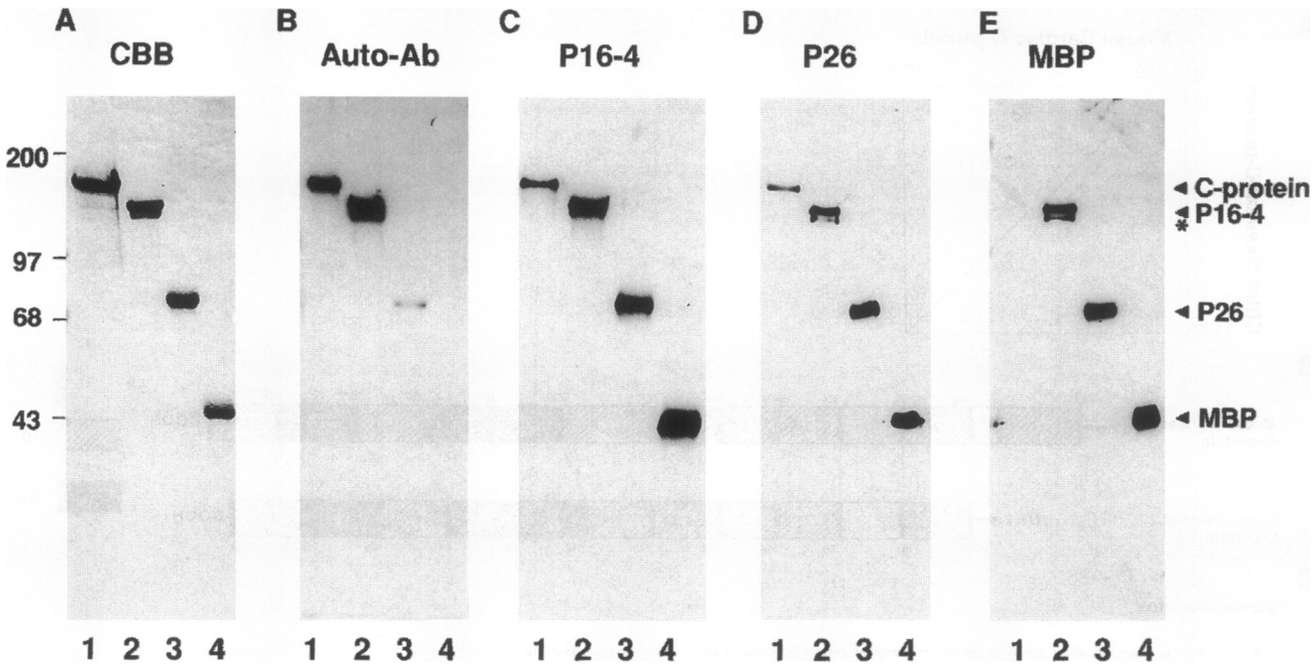

Figure 7. Immunoblot analysis of the fusion proteins. (*A*) Coomassie blue-stained gel of C-protein (*lane 1*), P16-4 (*lane 2*), P26 (*lane 3*), and MBP (*lane 4*). Accompanying immunoblots with the autoantibody (Ab 1) from mice immunized heart homogenate (*B*), from P16-4 immunized mice (*C*), P26 immunized mice (*D*), and MBP immunized mice (*E*). The autoantibody (Ab 1) reacts to P16-4 (*B, lane 2*) and reacts weakly to P26 (*B, lane 3*), but not to MBP (*B, lane 4*). The two autoantibodies against P16-4 and P26 reacted with C-protein (*C* and *D, lane 1*). These autoantibodies from fusion protein-immunized mice (*C–E*) react with all the fusion proteins (P16-4, P26, and MBP) by the crossreaction to MBP. The asterisk indicates degradation form of P16-4.

3 and 4) identity. Only domain 11 conserves a pair of cysteine in all 3 isoforms of C-protein, at this part the C-protein bound to myosin (37). In the three Fn-like domains, internal homology was similar to that of IgG-like domains between 19 (domains 7 and 10) and 26% (domains 8 and 10) identity.

Partial characterization of the antigenic peptide. We generated two fusion proteins, P26 (amino acid residue; 945–1270), and P16-4 (amino acid residue; 205–916) encoded by part of the cDNA, C26, and the PstI-PstI fragment of clone C16, respectively (Fig. 4 *B*). A full-length fusion protein could not be used as the antigen due to its low expression level and degradation in *E. coli.* As shown in Fig. 7 *B,* both fusion proteins P16-4 and P26 were recognized by the autoantibody (Ab 1). The stronger reactivity to P16-4 suggested that this fragment of C-protein has an autoantibody (Ab 1) recognition site. Thus we immunized purified fusion proteins and MBP to examine the pathogenicity of P16-4. As expected, P16-4 induced myocarditis in all of the immunized SMA mice at the dose of 100 μg and in 66% at the dose of 50 μg, whereas neither 100 nor 50 μg of P26 induced myocarditis (Fig. 8, Table II). In addition, both P16-4 and P26 produced antibodies against C-protein (Fig. 7, *C* and *D,* lane *1*); the former reacted strongly to C-protein. These findings suggested that the dominant immunogenic epitope exists in residues 205–916. As a control experiment, MBP alone did not produce the antibody against C-protein (Fig. 7 *E,* lane *1*) nor did it induce myocarditis (Table II). To confirm the antigenicity of this peptide 205–916 in other strains of mouse, we immunized DBA/1J (H-2^q), DBA/2J (H-2^d), BALB/c (H-2^d), C57BL/6 (H-2^b), O20/A (H-2^{pz1}), SJL (H-2^s), A/J (H-2^a), AKR/J (H-2^k), and C3H/He (H-2^k) with 100 μg of P16-4 which effectively induced myocarditis in SMA mice. All immunized DBA/1J, O20/A, and SJL mice developed myocarditis, whereas only a few C57BL/6 (25%), BALB/c (16%), C3H/He (16%), and no DBA/2J, A/J, or AKR/J developed myocarditis (Table II).

Demonstration of C-protein–reactive autoantibodies in DCM patients. We analyzed sera from 16 patients diagnosed with DCM, as well as sera from 83 normal individuals by immunoblotting against human heart homogenate (Fig. 9). No control sera (Fig. 9 *B*) and 2 of 16 DCM sera (Fig. 9 *A,* lanes *9* and *12*) reacted specifically to the protein which was ~ 150 kD. We confirmed that this 150-kD band was C-protein because these two sera reacted against the purified human cardiac C-protein (Fig. 9 *C,* lane *2*). Therefore, we conclude that 2 of 16 sera had an antibody to C-protein. In normal control sera, even though no sera preferentially recognized the 150-kD protein, we found a high incidence of autoantibodies against several human cardiac proteins including myosin, as Neumann et al. (7) reported previously.

Discussion

In this study, cardiac C-protein was found to be a dominant antigen among many types of cardiac proteins and to induce autoimmune myocarditis under experimental conditions. This conclusion is substantiated by the following experimental findings: (*a*) the autoantibody produced by the myocarditis-induced mouse specifically reacted to the 150-kD protein (C-protein) in spite of immunized whole cardiac tissue homogenate; (*b*) purified C-protein induced myocarditis; and (*c*) recombinant

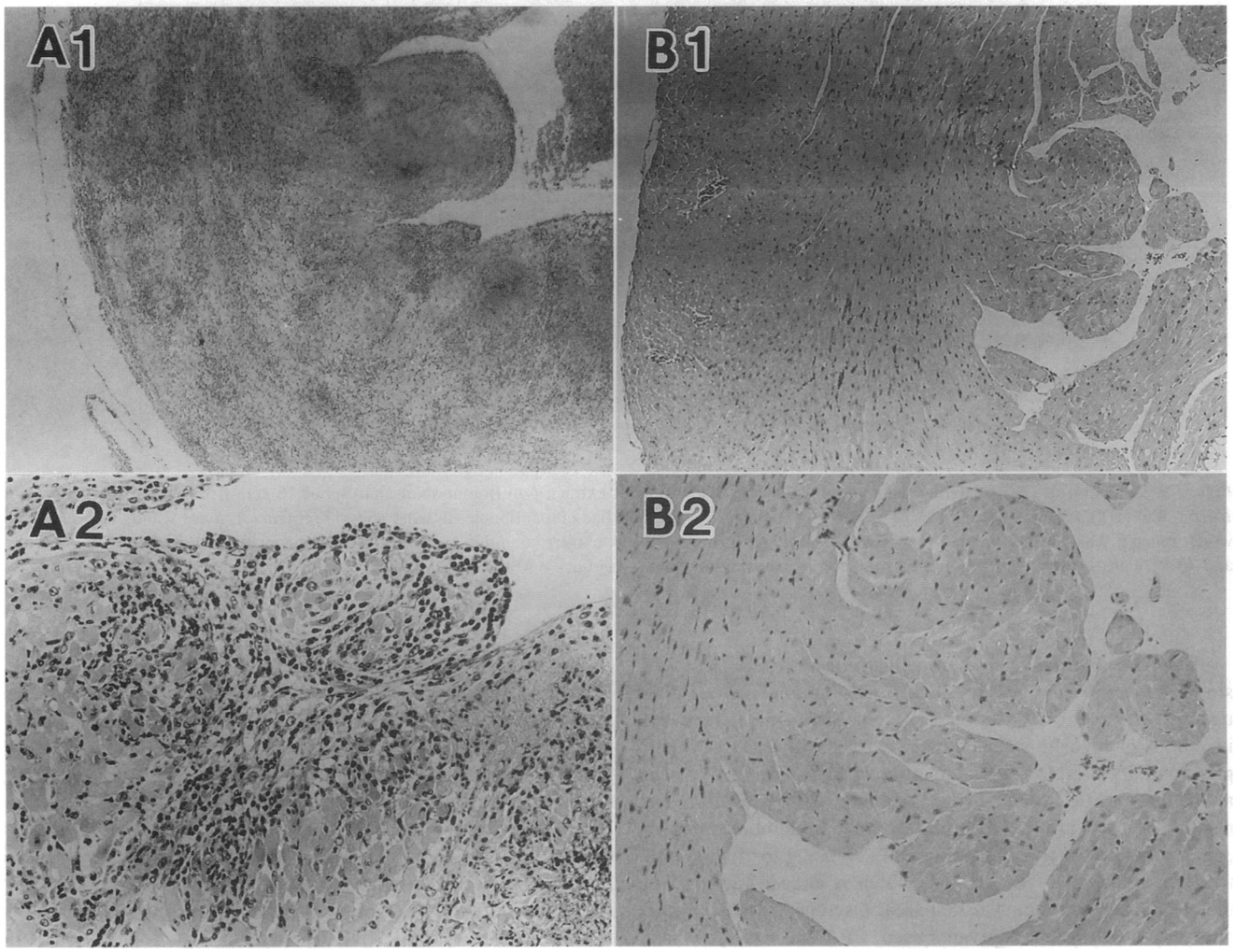

Figure 8. Autoimmune myocarditis induced by the repeated injections of recombinant proteins with KO3 LPS. (*A1* and *A2*) P16-4 induced severe inflammatory responses in the heart. No inflammatory changes were observed in mice immunized with P26 (*B1* and *B2*) (hematoxylin and eosin staining).

*Table II. Induction of Murine Autoimmune Myocarditis with Fusion Proteins**

Experiment	Mouse	Immunogen	Antigen dose × times	Induction of myocarditis
1	SMA	P16-4 (residue (205–916)	100 μg × 4	100% (6/6)
			50 μg × 5	66% (2/3)
		P26 (residue 945–1270)	100 μg × 5	0% (0/3)
			50 μg × 5	0% (0/3)
		MBP	100 μg × 5	0% (0/6)
			50 μg × 5	0% (0/6)
2	DBA/1J	P16-4 (residue 205–916)	100 μg × 5	100% (6/6)
	SJL			100% (6/6)
	O20/A			100% (5/5)
	A/J			0% (0/3)
	AKR/J			0% (0/3)
	Balb/c			16% (1/6)
	C3H/He			16% (1/6)
	C57BL/6			25% (1/4)
	DBA/2J			0% (0/3)

* Antigens mixed with 100 μg KO3 LPS were injected at 4-wk intervals.

cardiac C-protein (amino acid residue 205–916) effectively produced myocarditis in some mice strains including SMA, DBA/1J, SJL, and O20/A, but not in A/J, AKR/J, or DBA/2J mice strains.

In spite of the prevalence of autoimmune diseases, a limited number of autoantigens have been identified to induce disease in experimental animal models, e.g., thyroglobulin for chronic thyroiditis (44), acetylcholine receptor for myasthenia graves (45), uveal S antigen for uveitis (46), myelin basic protein for multiple sclerosis (47), type II collagen for synovitis (48), and gp330 and 44-kD complex for Heymann nephritis (49, 50). These autoantigens must be identified for the development of disease-specific therapeutic strategies, ultimately to human autoimmune disease (51, 52). The knowledge of the structure of the antigenic epitopes enabled us to use synthetic peptides that mimic pathogenic epitopes (53, 54). The present experimental findings showing that C-protein is an important antigen in the development of autoimmune myocarditis, and the partial identification of the pathogenic amino acid motif should help in the development of therapeutic strategies through the use of our myocarditis model.

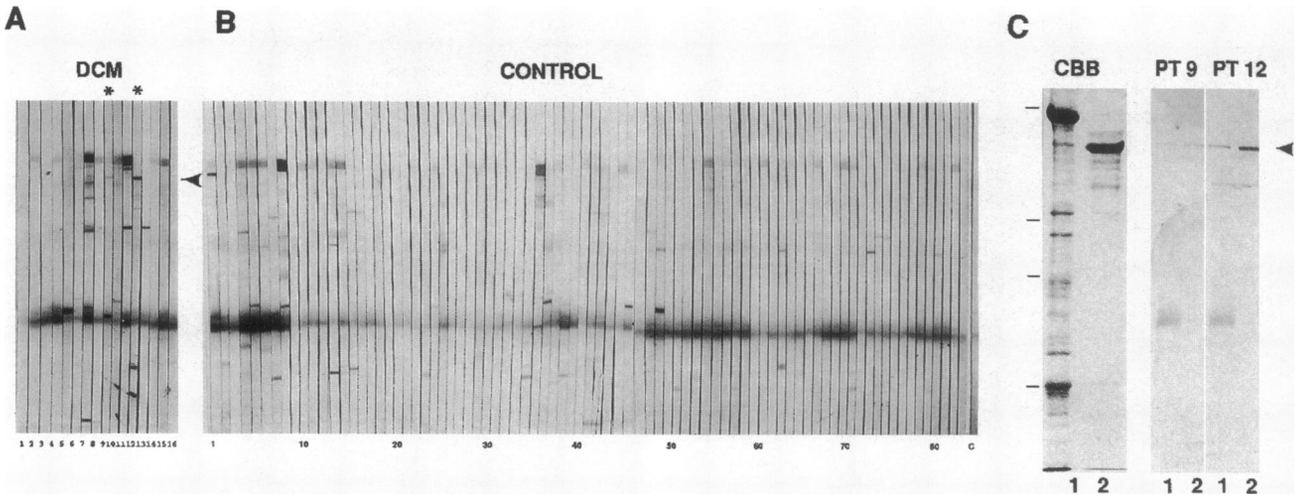

Figure 9. Detection of DCM patients' autoantibodies against human heart extract. (*A*) Immunoblot analysis of 16 sera from DCM patients (*lanes 1–16*), and (*B*) 83 normal sera (*lanes 1–83*). Two patients (*PT 9* and *12*) had autoantibodies against 150-kD protein (*A, lanes 9* and *12, asterisks*), which reacted with purified human C-protein (*C, lane 2*), as well as heart extract (*C, lane 1*). Positions of molecular mass markers (200, 97, 68, and 43 kD) are shown. Anti-human IgG reacted nonspecifically to the band at ~ 50 kD (*B, lane C*).

The present findings conflict with an earlier report that myosin is the major antigen producing autoimmune myocarditis under H-2 genetic restriction (12). In SMA mice, repeated injections of myosin-enriched heart extract with KO3 LPS still preferentially produced the autoantibody to C-protein. We also noted that purified cardiac myosin mixed with KO3 LPS did not induce myocarditis (data not shown). In addition, myosin contamination was avoided completely by using the fusion protein expressed in *E. coli,* P16-4, which is encoded by part of the cDNA of C-protein. This apparent discrepancy is explained partly by the different adjuvants used, CFA and LPS (12, 55). The other difference is observed in the responder strain. The A/J background is a high responder to myosin-induced myocarditis irrespective of the H-2 haplotype (12), and to CB3-induced myocarditis (56, 57). CB3-induced myocarditis, which might further trigger autoimmune reactions against myosin by a molecular mimicry mechanism, has been suggested to be a possible underlying pathogenesis of DCM (58, 59). In the present study, we suggest that the autoimmune triggering mechanism in C-protein–induced myocarditis may differ from a CB3-triggered or myosin-induced mechanism because: (*a*) crossreactive epitopes between CB3 and C-protein have not been reported; and (*b*) contrary to the previous results that A/J, C3H/He, Balb/c, DBA/2 strains have a high susceptibility to CB3 (56, 57), the C-protein residue 205–916 hardly induced myocarditis in these strains in the present experiment. The limitation of our approach is that we used a part of the C-protein, 60% of full length, for immunization; a full-length fusion protein could not be used as the antigen because of its low expression level and degradation in *E. coli.*

Further, it is of importance to attempt to explain the clear strain differences in the response to the C-protein mixed with LPS. SMA, DBA/1J (H-2^q), SJL (H-2^s), and O20/A (H-2^{pz1}) are high responders, whereas DBA/2J (H-2^d), BALB/c (H-2^d), C57BL/6 (H-2^b), A/J (H-2^a), AKR/J (H-2^k), and C3H/He (H-2^k) are poor responders. One explanation is the differing sensitivity to adjuvant KO3 LPS and, in part, the different repertoire of helper T cell receptor (TCR) used (60). However, the most important explanation of the association between the autoimmune disease and MHC alleles in human and animal models is that different peptides bind to different MHC II antigens; the triad formation between antigen, MHC, and TCR is critical to the activation of T cells and further immune responses (60).

The positions of the amino acid sequence responsible for the induction of myocarditis can be hypothesized. Little is known about the consensus sequence of amino acid motifs which bind to murine MHC II; only those for I-A^b, I-E^b (61), I-A^d (62), and I-A^s (63) have been reported. Here, in SJL mice, which express I-A^s, myocarditis was induced in 100% with fusion protein P16-4. C-protein has the consensus sequence which binds to I-A^s (63), VSXXXXR/H sequence (X can be any amino acid) derived from the transferrin receptor:

C-protein	FKCEVSDENVRGVWLK	(560–575)
	*** *	
Transferrin receptor	KPTEVSGKLVHANFGT	(203–218).

The pathogenic epitopes on the P16-4 in these four different strains remain to be determined.

Although myocardial cells are composed of many types of proteins, the mechanism by which the C-protein induces autoimmune myocarditis remains unknown. C-protein is one of the major constituent proteins necessary to compete with natural antigens such as invariant chain and MHC to obtain the position at the MHC antigen-binding site (64, 65). Further, examination of the structure of the C-protein leads to some speculation on its role in the autoimmune reaction. C-protein is an intracellular member of the immunoglobulin superfamily (36–38). IgG-like domains are conserved in most cell-surface immune recognition molecules (40), and also TCR and MHC class II have this domain (40). Recently, the peptides of recycled membrane proteins, class II HLA and invariant chain, have been shown to be major self-antigens which are presented by class II molecules

(61–63, 66, 67). However the HLA-binding motifs are located in the variable domain of this structure, not in this constant IgG-like domain (61–63, 66, 67). It may be questioned whether or not these IgG-like conformations work with each other at the cell surface similar to the directly bound MHC-TCR like superantigen (68). These questions remain to be answered.

Finally, the possible involvement of C-protein–triggered autoimmune myocarditis in DCM must be discussed. In this study, it was found that the sera of 2 DCM patients of the 16 preferentially recognized the C-protein, as based on the results of immunoblotting against whole cardiac proteins. On the other hand, none of the control sera specifically reacted to the C-protein. In spite of numerous studies, the pathogenesis of DCM still remains obscure, and treatment is generally cardiac transplantation. Considering the DCM is a pluricausal disease (3) and 2 of 16 patients' sera specifically recognized the C-protein, we hope this experimental model will lead to the elucidation of one of the possible causes of this disease. Also, if autoimmune reaction occurring by molecular mimicry between CB3 and myosin or C-protein is one of the causes of DCM, it would be of interest to test the changes in autoantibody production in the chronic stage of infectious myocarditis. To clarify this point, sera from patients with acute and chronic phase myocarditis would be needed.

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