Dilated Cardiomyopathy with Special Reference to Humoral Immunity

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The question of whether the etiology of DCM is immune or autoimmune has been increasingly discussed. Abnormal findings on humoral immunity in DCM were investigated, especially those regarding anti-heart antibodies (AHA), IgG subclasses and soluble interleukin-2 receptor (sIL-2R). The heterophile type AHA was detected in 64.7% of cases by the indirect immunofluorescence technique (IF) with rat heart, by indirect IF with human heart AHA in 57.8% of cases, and by thin-layer chromatogram with human glycolipids AHA in 44% of cases. Also, 57.1% of the specimens were found to bind IgG on pericytes by direct IF with biopsy specimens taken from patients with DCM. The epitope of an antigen which reacted with the heterophile type AHA is a Gal α 1-3Gal structure. 200 Kd, 70 Kd and 40 Kd antigens were reacted with AHA detected by indirect IF with human heart. The possible mechanisms of AHA in the pathogenesis could be either complement dependent cytotoxicity or interference to cardiac metabolism. The concentration of sIL-2R and IgG3 in sera from patients with DCM were elevated. These results suggest that immunological abnormalities occur continuously in DCM. (Jpn Circ J 1992; 56: 1073-1080)

Dilated cardiomyopathy (DCM), a heart muscle disease of unknown etiology, is characterized by ventricular dilation and a reduction in systolic performance. Although the etiology of DCM remains unknown in the majority of patients, recently there has been increasing evidence from both clinical and experimental studies, that this syndrome is a sequela of viral myocarditis. Recently, the possible role of viral etiology, not only of the initial insult, but also of the ongoing disease process, has been resurrected, because by means of in vitro nucleic acid hybridization as well as by the use of the polymerase chain reaction, echovirus RNA has been detected in the myocardium of a significant number of patients with DCM. However, because abnormalities in both cellular and humoral immune function in patients with DCM have been demonstrated, an immune or autoimmune etiology of DCM has also been considered. In this paper, we show abnormal findings in the humoral immunity of patients with DCM especially regarding anti-heart antibodies (AHA) and the serum concentration of the soluble interleukine-2 receptor (sIL-2), as well as IgG subclasses.

MATERIALS AND METHODS

Serum samples
Circulating AHA reacting with rat heart as

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detected by indirect immunofluorescence technique (IF) were examined in the sera of 31 patients with DCM (mean age of 49.3 years). Circulating AHA reacting with human heart as detected by indirect IF were examined in the sera of 64 patients with DCM (47.2 years). These patients sera were supplied by the members of the Research group for Epidemiology and Etiology of Idiopathic Cardiomyopathy, the intractable diseases of the Ministry of Health and Welfare in Japan. Circulating antibodies against glycolipids of the human heart detected by thin-layer chromatogram (TLC) immunostaining were examined in the sera of 23 patients with DCM (47.0 years). Binding antibodies to the human heart detected by direct IF were examined in the biopsied materials of 14 patients with DCM (59.8 years).

The concentrations of IgG subclasses were measured in sera of 9 patients with DCM (54.7 years), 10 with AMI (61.1 years) and 8 of normal controls (40.3 years).

The concentration of sIL-2 was measured in sera of 8 patients with DCM (50.4 years) and 5 of normal controls (47.4 years).

Detection of AHA

We used four different approaches to detect AHA as follows;

1) Indirect IF with rat heart as antigen

An indirect IF was carried out using the modified method of Choi and Reiner. A rat heart ventricle was cut into small blocks, fixed in cold 95% ethanol, and embedded in paraffin at 58 °C. Sections were cut at 4 μm and deparaffinized. The slides were washed for 15 min in 0.01 M phosphate buffered saline (PBS, pH 7.2) 3 times. The serum samples were diluted to 1: 10 with PBS, placed on the section, and incubated for 50 min in a moist chamber at room temperature. After washing 3 times in PBS, the specimens were incubated for 30 min with a 1: 10 FITC conjugated rabbit anti-human IgG serum. The sections were then washed for 15 min 3 times in PBS, mounted in 90% glycerol at pH 8.4 and viewed under a Leitz fluorescent microscope. The staining patterns of the AHA were classified into 4 types namely, heterophile, intercellular, intercalated disc and nuclear as reported previously.

The analysis of an epitope of the antigen against the heterophile type AHA was carried out with two heterophile type AHA positive sera from patients with DCM and with two control sera which were AHA negative. Glycolipids that bind the antibody were detected by thin-layer chromatogram (TLC) immunostaining using Magnani’s method with slight modifications. The purified glycolipids used as antigens were ceramide monoheixoside (GalCel), ceramide dihexoside (LacCel), ceramide trihexoside (Gb3Cer), globoside (Gb4Cer), asialo GM1 (Gb3Cer), paragloboside (nLe6Cer), Fossuman antigens (IV3GalNAc α Gb4Cer), α-galactosylparagloboside (IV3Gal α nLe6Cer), Hangutziu-Deicher (HD) antigen (II3NeuGc-LacCer). These glycolipids were kindly supplied by Masao Iwamori M.D., University of Tokyo, Tokyo, Japan. These glycolipids were chromatographed on aluminum-backed high-performance TLC plates (Silica Gel 60; E. Merk, Darmstadt, Federal Republic of Germany) in chloroform-methanol-water (CMW, 60: 35: 8, v/v). The plate was then overlaid with serum. After washing with PBS, the plate was overlaid with 125I labeled goat anti-human IgG serum. Then the plate was washed and exposed to XAR-5 X ray film. Indirect IF was carried out with sera absorbed with the purified glycolipids and human red blood cells (type A, B, AB and O).

2) Indirect IF with human heart as antigen

A human heart obtained at autopsy was frozen until use. The method of the staining was the same as mentioned above.

Analysis of the antigens reacting with the AHA was carried out by Western immunostaining. Human myocardium was homogenized using a Polytron tissue homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min. The resulting supernatant fluid was subjected to ultracentrifugation at 100,000 x g for 60 min. The pellet was re-homogenized and referred to as the membrane fraction. Sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAG) electrophoresis was carried out according to the method of Laemmli. Aliquots of the membrane fraction of the myocardium were mixed with an equal volume of SDS-PAG electrophoresis sample buffer, heated at 100 °C for 5 min and centrifuged for 5 min at
15,000 rpm. A 200 µl sample (6.3 µg/µl) was loaded onto a 4% to 20% linear gradient PAG minigel. Electrophoresis was performed at 100 mA. A small piece was cut from the end of the gel and stained with Coomassie blue to evaluate the efficacy of the separation. The remainder of the gel was placed on a piece of Immobilon PVDF transfer membrane (issenprotech, MA, U.S.A.). Blotting was performed with ISS SEMI-DRY ELECTRO BLOTTER (issenprotech, MA, U.S.A.). The membrane was immersed in PBS and was subsequently cut into 2–3 mm wide strips for immunostaining. The position of each molecular weight marker on the nitrocellulose strip was measured relative to the origin and to the bottom of the gel. The acrylamide concentration at the position of each marker was estimated by linear interpolation. A molecular weight standard curve based on the log acrylamide concentration and log molecular weight of each marker was determined for the gel.

3) TLC immunostaining with glycolipids (GLs) extracted from human heart as antigen

GLs were extracted from human myocardium as follows. Myocardium was taken from autopsied materials, and was homogenized in 3 volumes of iced distilled water. The homogenate was then poured into 10 volumes of methanol followed by the addition of 5 volumes of chloroform under constant stirring for 30 min at room temperature. After centrifugation at 2000 rpm for 10 min, the supernatant was saved, and the pellet was resuspended in 4 volumes of distilled water, followed by the addition of 15 volumes of CM (1:2, v/v). The mixture was centrifuged again for 10 min at 2000 rpm and supernatants were combined. The supernatant was dried and resuspended in 5 volumes of CMW (60:35:4.5, v/v). GLs were separated into neutral and acidic GL using ion-exchange chromatography. The lipid extracts were desalted by chromatography on Sephadex G-25. Desalted GLs dissolved in CMW (30:60:8, v/v) with ammonium acetate were loaded on a column of ionic form DEAE-Sepharose. Neutral glycolipids were then eluted with 2 column volumes of the same solvent followed by 10 column volumes of methanol. Acidic glycolipids were eluted with 10 column volumes of 0.5 M ammonium acetate in methanol. The TLC immunostaining with these GLs were carried out as mentioned above.

4) Direct IF was carried out as follows in order to bind antibodies on the myocardium of patients with DCM

Criostat sections of 4 µm of biopsied specimens were washed in cold PBS and incubated with FITC-labeled anti-human IgG rabbit serum for 45 min.

Effect for myocardium of AHA binding

Myocytes derived from newborn rats were cultured according to the method of Harady and Farley. Rats, 2–4 days old, were killed, and the hearts were removed rapidly. After washing in Ca-free and Mg-free cold PBS, the ventricles were cut into about 1 mm³ fragments, and suspended in 0.125% trypsin dissolved in EDTA solution. The ventricles were stirred gently for 10 min, at 37 °C, then filtered through mesh and suspended in Eagle’s minimum essential medium (MEM) containing 10% inactivated fetal calf serum (FCS), at 4 °C. The collected supernatant was centrifuged at 800 rpm, for 5 min. The pellet was resuspended in Eagle’s MEM containing 10% FCS. Approximately, 1.5–2×10⁵ cells/ml were placed in a Falcon plastic dish (35 mm diameter.) After culturing for 90 min at 37 °C in an incubator with 5% CO₂ and 95% O₂, non-adherent cells were collected and used as myocytes.

Circulating antibodies against the cultured myocytes were produced by injection of the myocytes (5×10⁶) intravenously into a normal rabbit. The animals were immunized five times in every week. The animals were bled 1 week after the final injection. The control serum was obtained from a rabbit injected with MEM using the procedure described above.

Evaluation of cytotoxic activity of the circulating AHA

3×10⁴ Myocytes/well of a Falcon 3074 culture plate were labeled with ⁵¹Cr (3 µCi/well) for 12 h. Labeled myocytes were washed three times, and then incubated with the inactivated sera (1:1, v/v) at 4 °C
for 45 min. After washing, the contents of the wells were added to fresh normal rabbit's complement and incubated at 37°C for 2 h. After centrifugation at 300 rpm for 10 min., radioactivity was estimated using a gamma counter. The cytotoxic activity was estimated by the following formula: % 51Cr release = cpm (experimental) - cpm (spontaneous release)/cpm (maximal) - cpm (spontaneous release) × 100. The values are presented as the mean of triplicate samples.

**Determination of cAMP concentration in myocytes**

1×10^6 of myocytes, cultured in a Falcon 30 mm dish for 2 days, were incubated with the inactivated sera (1:1, v/v) at 4°C for 30 min. After washing, they were incubated with MEM for 1 h in a CO2 incubator. After washing, they were centrifuged at 5000 rpm for 2 min, after which the cell pellets were homogenized with 0.5 ml of 0.1 M HCl at 13000 rpm for 1 min. After centrifugation, the cAMP concentration in the supernatants was determined by a YAMASA cAMP Assay Kit.

**Measurement of IgG subclass concentration**

IgG subclass concentration was measured with the enzyme-linked immunosorbent assay with each monoclonal antibodies. Namely, microtiter plate surfaces were coated directly with subclass-specific monoclonal antibodies. Following the addition of serum samples or standards, the bound immunoglobulin subclass was detected with horseradish peroxidase anti-human IgG conjugates. The reaction was terminated by addition of the stop solution, and absorbance at 490 nm was measured.

**Measurement of sIL-2R concentration**

The quantitative determination of sIL-2R levels in serum was carried out with CELL-FREE interleukine-2 Receptor Test Kit (T cell Science, Inc). Briefly, an anti IL-2R monoclonal coating antibody is first absorbed onto polystyrene microtiter wells. Soluble IL-2R present in the sample or standard binds to the antibody on the coated well; unreacted sample components are removed by washing. An enzyme conjugated anti IL-2R monoclonal antibody directed against a second epitope on the IL-2R molecule is then added and binds to the IL-2R captured by the first antibody, completing the sandwich. Unbound enzyme-conjugated anti-IL-2R is removed during a wash step and substrate solution is added to the wells. A colored product is formed in proportion to the amount of IL-2R present in the sample. The reaction is terminated by
addition of stop solution, and absorbance at 490 nm is measured.

RESULTS

I: Prevalence of Anti-heart antibodies in serum from patients and on biopsy specimens from patients with DCM

By indirect IF with rat heart, the heterophile type of AHA was detected in 22 out of 32 sera from patients with DCM (64.7%). By indirect IF using human heart, the AHA was detected in 37 out of 64 sera with DCM (57.8%). By TLC immunostaining with human GLs, 11 out of 25 sera with DCM (44.0%) were seen to contain AHA. By direct IF with biopsy specimens taken from DCM patients, 8 out of 14 specimens (57.1%) were seen to bind IgG on perimyo-

cytes.

II: Analysis of antigen reacted with AHA

The epitope structure of the antigen reacting with heterophile type AHA was analy-
ized. By TLC immunostaining with purified glycolipids and heterophile type AHA positive or negative sera, the positive sera was seen to bind with α-galactosyl-paragloboside and ceramide monohexoside, while the negative sera bound only with ceramide

monohexoside.

By indirect IF with the absorbed sera, the heterophile type staining completely disappeared with the sera absorbed with α-galactosylparagloboside, while sera absorbed with CMH showed no change in the staining pattern. By comparing the structures of used glycolipids that fail to bind to the antibody, the epitope that reacted against the heterophile type AHA was deduced as terminal Gal α 1-3Gal as shown in Fig. 1.

The antigens reacting with perimyocyte type AHA detected by indirect IF technique with human heart were analyzed by Western immunostaining for IgG activity against normal human myocyte membrane extracts. As shown in Fig. 2, staining bands of 200 kilodalton (Kd), 70 Kd and 40 Kd appeared. The prevalence of these staining bands were 61.5%, 23.0% and 69.2%, respectively. The physiological function of AHA;

1) Cytotoxic activities: Fig. 3 shows the results of the complement-dependent cyto-
toxic tests against cultured rat myocytes. In the assay system with complement, the CI values using AHA positive sera (n=8) were 72.2±22.5% (mean±SD), whereas those
Fig. 5. The levels of IgG subclasses in sera from patients with dilated cardiomyopathy or acute myocardial infarction and from normal controls.

Fig. 6. The levels of soluble IL-2R in sera from patients with dilated cardiomyopathy and from normal controls. Stippled area shows normal levels.

using AHA negative control serum were $8.0 \pm 4.3\%$. Those values did not decrease after the sera was absorbed with non-myocytic cells in culture dishes.

2) The effects of cAMP concentration in the myocytes treated with AHA: As shown in Fig. 4, the concentration of the cAMP in the myocytes treated with the AHA positive sera were $7.4 \pm 2.0 \, \text{pmol/10}^6 \, \text{cells}$ (mean $\pm$ SD), whereas the concentration in those treated with the AHA negative sera or with MEM were $3.9 \pm 1.0 \, \text{pmol/10}^6 \, \text{cells}$ and $3.5 \pm 1.4 \, \text{pmol/10}^6 \, \text{cells}$, respectively.

The concentrations of IgG subclasses in each group is shown in Fig. 5. The level of IgG3 in DCM was higher than in AMI and normal controls.

The concentration of sIL-2R is shown in Fig. 6. The levels of sIL-2R were significantly higher in DCM than in normal controls.

**DISCUSSION**

This investigation presents abnormalities in an aspect of humoral immunity. Namely, the presence of circulating anti-heart antibodies, the presence of antibody-bound myocytes in biopsy specimens from DCM patients, the abnormal distribution of IgG subclasses and elevation of sIL-2R concen-
tion.

The presence of several kinds of antibodies which reacted with myocyte in serum from patients with DCM was demonstrated. Interestingly, IgG bound to myocytes was found in 57.1% of biopsy specimens from patients with DCM. Several specific autoantibodies which may contribute to ongoing DCM were reported, for example antibodies to the β adrenoceptor! to adenine nucleotide translocator (ANT) to a second mitochondrial enzyme, branched chain keto acid dehydrogenase. We have also reported that heterophile type AHA has a capacity for complement dependent cytotoxic activity. As shown in this report, the epitope structure of the antigen reacted with the heterophile type AHA is Gal α 1-3Gal, so these antibodies are the so-called α Gal antibodies. Furthermore, 200 Kd and 40 Kd antigens in the cell membrane were detected. These molecular weights were similar to those of myosin and actin, respectively. Further characterization of these antigens is necessary, because myosin is one of the candidates for antigen-inducing auto-immune myocarditis.

Studies in mice suggest that autoimmune myocarditis occurred in genetically susceptible strains as a sequela to viral myocarditis. Whether the mechanism involved in autoimmune myocarditis is cellular immunity or humoral immunity is also dependent on genetic susceptibility. A study undertaken in collaboration with Nishi et al., Kurume University, Japan, showed that a difference in genetic markers between DCM patients with or without AHA exists, as mentioned in the symposium reports by Nishi et al.

Two kinds of effects of AHA on myocytes was found in this study, namely complement-dependent cytotoxicity and cardiac function. Antibodies to ANT interfered with cardiac function and antibodies to β adrenoreceptor also had an influence on the level of cardiac adenylate cyclase.

Recently, several new immunological markers have been used to measure immunological activity in patient's serum. One of them is a concentration of the IgG subclass. The association of specific antibody activities with particular subclasses was reported. Interestingly, the subclass response to viral infections is mainly restricted to IgG1 and IgG3. IgG1 and IgG3 were also elevated in patients with autoimmune disease. In our study, an elevation of IgG3 subclass level was found in DCM patients. Further studies are needed to clarify the mechanism of elevation of IgG3 subclass in DCM.

Another of these markers is a sIL-2R concentration. IL-2 (T cell growth factor) exerts effects through interaction with cell surface receptors. Recently, a sIL-2R concentration was detectable in patient's serum. As the generation of sIL-2R appears to be an inevitable consequence of immune activation in vitro elevated levels of serum IL-2R might be found in association with immune activation in vivo, as in heart transplantation. In this regard, the elevation of sIL-2R concentration in the serum of patients with DCM is one of markers of abnormal immune activation.

Further studies are necessary to analyze immunologically heterogeneity of DCM and to develop better criteria for diagnosis of immunologically-mediated DCM.

REFERENCES


7. MAGUNANI JL, NILSSON B, BROCKHAUS M, ZOPF D, STEPELEWSKI Z, KOPROWSKI H, GINSBURG V: A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-n-fucopentaose II. J Biol Chem 1982; 257:


20. YOUNG JB, WINDSOR NT, KLEIMAN NS, SMART-FW, COCANOUGH B, LAWRENCE C: Relationship of persistently elevated soluble interleukin-2 levels after heart transplant to allograft arteriopathy. *Am Coll Cardiol* 1991; **17**: 102A