In Situ Analysis with Monoclonal Antibodies of Lymphocyte Subsets in Myocardial Biopsies from Patients with Dilated Cardiomyopathy and Idiopathic (Viral) Myocarditis

HIROFUMI DEGUCHI, M.D., TETSUYA HAYASHI, M.D.

MASAHIRO KOTAKA, M.D., YASUSHI NAKAYAMA, M.D.

YASUSHI KITaura, M.D., AND KEISHIRO KAWAMURA, M.D.

This light- and electron-microscopic immunohistochemical study using monoclonal antibodies analyzes of the in situ lymphocyte subsets in endomyocardial biopsies from 11 patients with dilated cardiomyopathy (DCM) and three patients with idiopathic (viral) myocarditis (MYO). In the DCM patients both Leu 2a⁺ cytotoxic/suppressor T cells (Tc/s) and Leu 3⁺ helper/inducer T cells (Th/i) were identifiable in the myocardial lesions, and mean Th/i/Tc/s (T4/T8) ratio was 0.7 ± 0.6 (mean $\pm$ SD). In 9 of the 11 DCM patients, Tc/s were more numerous than Th/i cells, so the T4/T8 ratio was less than 1.0. On the other hand, the T4/T8 ratios varied widely in the three MYO patients; one of them had marked mononuclear cell infiltrates with many Th/i in the inflammatory foci and a T4/T8 ratio of 2.6. Immunoelectron microscopy revealed some Th/i in close contact with macrophages. These T cells in the myocardium of DCM and MYO patients appeared to be in vivo effector cells playing an important role in cell-mediated immune responses.

THE pathogenesis of dilated cardiomyopathy (DCM) and of myocarditis appears to be multifactorial¹. Several authors have suggested that cell-mediated immunity is involved in the pathogenesis of these diseases,²⁻⁶ and various degrees of mononuclear cell infiltration have been documented in the endomyocardial biopsies of patients with DCM^{5,7-9}. Zee-Cheng et al. stated that lymphocyte infiltrates in the myocardium may be more common than previously suspected and there may be ongoing inflammation in many cases of DCM⁵. Therefore, increas-

ing interest has focused on the immunological role of the lymphocyte infiltrates in the myocardium in patients with myocarditis and with DCM. In experimental coxsackie B virus myocarditis we have employed immunohistochemical analysis of T lymphocyte infiltrates in myocardial necrotic lesions and have suggested that these T cells play an important role in the immune response in myocarditis.^{10,11} This type of in situ analysis of lymphocyte subsets in the myocardium may aid the immunological study of patients with myocarditis and with DCM.

The present light- and electron-microscopic immunohistochemical study provides information on the tissue distribution as well as the relative proportion of T lymphocyte subsets in myocardial biopsies obtained from patients with

Key words:

T lymphocyte
Dilated cardiomyopathy
Myocarditis
Myocardial biopsy
Immunohistochemistry

Third Division, Department of Internal Medicine, Osaka Medical College, Takatsuki, Osaka, Japan

This study was supported in part by a Grant-in-Aid for developmental Scientific Research from the Ministry of Education, Science and Culture, and a Research Grant for Intractable Diseases from the Ministry of Health and Welfare of the Japanese Government.

Mailing address: Hirofumi Deguchi, M.D., Third Division, Department of Internal Medicine, Osaka Medical College, 2-7, Daigaku-cho, Takatsuki City, Osaka 569, Japan

TABLE I CLINICAL AND HEMODYNAMIC FINDINGS

Pt	Age (yr) Sex	NYHA	Duration after onset	CTR (%)	EDVI (ml/m ²)	CI (L/min/m ²)	EF (%)	Location of biopsy
<i>DCM</i>								
1.	59 F	III	10 years	52	125	3.14	29	LVB
2.	35 F	IV	1 month	75	153	2.68	27	LVB
3.	52 M	II	2 years	47	94	2.74	54	LVB
4.	53 M	III	10 years	53	151	4.10	42	RVB
5.	48 M	IV	10 months	55	73	3.99	73	RVB
6.	48 M	IV	2 years	61	91	3.05	38	RVB
7.	64 M	IV	50 days	55	93	2.92	38	LVB
8.	70 M	IV	3 months	55	82	2.30	34	RVB
9.	59 M	IV	3 years	52	96	3.00	32	RVB
10.	52 M	I	1 month	54	105	2.16	43	RVB
11.	53 M	III	5 months	48	100	3.11	38	RVB
<i>MYO</i>								
12.	52 M	I	30 days	45	65	2.14	49	RVB
13.	22 F	IV	40 days	60	85	5.15	66	RVB
14.	57 M	I	30 days	52	84	3.20	57	RVB

Abbreviations: CI = cardiac index; CTR = cardio-thoracic ratio; DCM = patients with dilated cardiomyopathy; EDVI = end-diastolic volume index; EF = ejection fraction; LVB = left ventricular biopsy; NYHA = NYHA functional classification; MYO = patients with viral or idiopathic myocarditis; RVB = right ventricular biopsy

DCM and idiopathic (viral) myocarditis (MYO).

MATERIALS AND METHODS

Patients Studied

Eleven patients with DCM (patients 1–11) and 3 patients with MYO (patients 12–14) who underwent diagnostic cardiac catheterization between March 1984 and January 1986 were studied. Systemic hypertension, valvular heart disease, coronary artery disease and congenital disease had been excluded by thorough cardiac examination, including coronary cine-angiography and echocardiography. The three MYO patients had clinical manifestations suggestive of a viral illness, such as flu-like or gastrointestinal symptoms, several days prior to the onset of cardiac symptoms, but serologic tests for viral infection were not positive. Table I summarizes the clinical, laboratory and hemodynamic findings.

Myocardial Biopsies and Tissue Preparations

The biopsies were obtained from the right or left ventricle by Konno-Sakakibara's bioprobe during cardiac catheterization. Specimens were immediately cut into two or more pieces measur-

ing 0.5 to 1.0 mm³ with a razor blade and used for light- and electron-microscopic immunohistochemical and conventional light- and electron-microscopic observations.

Light- and Electron-microscopic Immunohistochemistry

The biopsy specimens were fixed in a periodate-lysine-paraformaldehyde fixative (PLP), originally described by McLean and Nakane,¹⁵ and then washed in phosphate-buffered saline (PBS) containing 10% sucrose at 4°C for 2 hours and placed in OCT (Tissue Tek II, Lab TEK products, Division of Miles Laboratories, Naperville, IL, USA). They were immediately quick-frozen in a liquid nitrogen bath and stored at -70°C until processed. Frozen sections (6–8 µm) were cut and mounted on albumin glass slides. They were air-dried at room temperature, washed in PBS and then incubated at 4°C overnight with diluted anti human-monoclonal antibodies, which anti-Leu 2a or Leu 3a+b antibodies (Leu series, Becton Dickinson Monoclonal Center, Inc. Sunny Vale, CA, USA). After an additional washing with PBS, the sections were incubated for 45 min with peroxidase conjugated goat F(ab')₂ anti-mouse IgG antibody (Tago, Inc.

Fig.1. Light microscopic immunohistochemistry of endomyocardial biopsy from a 59-year-old female with DCM. Anti-Leu 2a antibody and peroxidase conjugated goat F(ab')₂ anti-mouse IgG antibody were used to identify cytotoxic/suppressor T cell infiltrates in the myocardium. A Leu 2a⁺ lymphocyte can be identified by red-brown reaction products on their membranes (arrowhead). Counterstained with hematoxylin. Scale, 20 μ m.

Fig.2. Light microscopic immunohistochemistry of endomyocardial biopsy from a 52-year-old male with idiopathic myocarditis stained with anti-Leu 3 antibody and peroxidase conjugated goat F(ab')₂ antimouse IgG antibody. Some helper/inducer T lymphocytes are identifiable in the myocardium. Counterstained with hematoxylin. Scale, 20 μ m.

TABLE II NUMBERS OF LYMPHOCYTES AND PERCENTAGES OF T CELL SUBSETS IN MYOCARDIAL BIOPSIES

Pt	Number of lymphocytes per unit area (0.2 mm ²)	Percentages of T cell subsets among total mononuclear cells		
		Leu 2a ⁺ (%)	Leu 3 ⁺ (%)	Leu 3 ⁺ /Leu 2a ⁺
DCM				
1.	7	16.4	8.5	0.52
2.	5	3.9	1.5	0.38
3.	7	7.9	5.6	0.71
4.	11	10.4	3.1	0.30
5.	8	8.6	4.7	0.55
6.	6	9.2	2.7	0.29
7.	9	8.4	13.3	1.58
8.	14	8.1	1.6	0.20
9.	11	9.2	8.7	0.95
10.	4	10.9	4.2	0.39
11.	6	3.0	6.7	2.23
mean ± SD	8 ± 3	8.7 ± 3.5	5.5 ± 3.6	0.74 ± 0.63
MYO				
12.	35	5.3	13.4	2.53
13.	13	12.9	1.4	0.11
14.	22	24.0	18.5	0.77
mean ± SD	23 ± 11	14.1 ± 9.4	11.1 ± 8.8	1.14 ± 1.25

Abbreviations: Pt = patients; DCM = patients with dilated cardiomyopathy; MYO = patients with viral or idiopathic myocarditis; mean ± SD = mean ± standard deviation

Burlingame, CA, USA).

For light microscopy, the sections were treated with diaminobenzidine (DAB)-peroxide solution for 4–8 min and counterstained with Mayer's hematoxylin. Stained sections were mounted in Permount (Fisher Scientific Company, NJ, USA) and viewed under a light microscope.

For immuno-electron microscopy, the incubated sections were postfixed with 2% glutaraldehyde, allowed to react with DAB for 30 min and DAB peroxide for 3 min and fixed with 1% osmium tetroxide solution for 30 min. They were then dehydrated in graded ethanols and embedded in epon. Ultrathin sections were examined without additional staining with a Hitachi-H 800 electron microscope (75 kV).

For control staining, all immunoperoxidase procedures included both positive and negative control slides. Positive controls for the specificity of the monoclonal antibodies used in this study consisted of lymph node tissue obtained from patients with neither heart disease nor immune-mediated disease.

T-Lymphocyte Enumerations in Frozen Sections

In the frozen sections stained with monoclonal antibodies, T lymphocytes were identified under the light microscope by red-brown reaction products on their surface or within their cytoplasm. In the light-microscopic section from each biopsy, interstitial cells, including macrophages, lymphocytes, polymorphonuclear leukocytes, plasma cells and fibroblasts, were enumerated consecutively in a total cell count of 50 to 500 at ×500 magnification. The percentage of Leu 2a and Leu 3a+b antigen positive cells in the total interstitial cell population was calculated. The mean lymphocyte count and standard deviation were obtained in each patient group.

Conventional Light Microscopic Observations

For conventional light microscopy, each myocardial biopsy was fixed in 10% formalin, dehydrated with a series of ethanols and embedded in paraffin. Paraffin-embedded sections of biopsies were stained with hematoxylin and

Fig.3. Immuno-electron micrograph of endomyocardial biopsy from a 52-year-old male with DCM stained with anti-Leu 2a antibody and peroxidase-conjugated goat F(ab')₂ anti-mouse IgG antibody. Electron-opaque DAB reaction products are deposited focally along the surface membrane of a cytotoxic/suppressor T lymphocyte (Tc/s). MC, myocardial cell. No staining with uranyl acetate or lead citrate. Scale, 1 μ m.

eosin, Mallory-azan, elastica-van-Gieson and PAS, and examined by light microscopy.

For the enumeration of infiltrating lymphocytes, the paraffin-embedded sections of myocardial biopsies stained with hematoxylin and eosin were examined under a light microscope with a projecting screen. Visual fields corresponding to 0.01 mm² each projected consecutively onto the screen fitted with a unit grid. The number of lymphocytes in each field was counted at $\times 1000$ under a $\times 100$ oil-immersion objective. In one tissue section per biopsy, 20 fields were surveyed, so that the number of lymphocytes per 0.2 mm² was enumerated for each biopsy.

RESULTS

(1) Light Microscopic Immunohistochemistry in Myocardial Biopsies

In the frozen sections stained with the indirect immuno-peroxidase method using monoclonal antibodies and counterstained with Mayer's hematoxylin, cardiocytes and mononuclear cell infiltrates were well preserved and easily recog-

nizable. Immunohistochemical examination of the myocardial biopsies from DCM patients revealed some Leu 2a⁺ cytotoxic/suppressor (Fig. 1) and Leu 3⁺ helper/inducer T lymphocytes in the myocardial lesion. The percentages of these Leu 2a⁺ cytotoxic/suppressor and Leu 3⁺ helper/inducer T lymphocytes among all the interstitial cells were $8.7 \pm 3.5\%$ and $5.5 \pm 3.6\%$ (mean $\pm$ SD), respectively (Table II). In the 11 patients with DCM, cytotoxic/suppressor T cells were often more numerous than helper/inducer T cells in the myocardium, so that the ratio of Leu 3⁺ cells/Leu 2a⁺ cells (T4/T8) averaged 0.74 ± 0.63 , far less than 1.0. There was no statistical significant correlation between T4/T8 ratios and various clinical data, including NYHA functional classes and hemodynamic data.

In our three MYO patients the T4/T8 ratios varied widely. Patient 12 (M.Y. 52 year-old) had many mononuclear cells in the interstitium (35 lymphocytes per 0.2 mm²). Immunohistochemical study revealed more Leu 3⁺ helper/inducer T cells than Leu 2a⁺ cytotoxic/suppressor cells (Fig. 2), so that the T4/T8 ratio was 2.53 (Table II). The other two MYO patients had

Fig.4. Immuno-electron micrograph of endomyocardial biopsy from a 52-year-old male with idiopathic myocarditis stained with anti Leu 3 antibody and peroxidase-conjugated goat F(ab')₂ anti-mouse IgG antibody. A helper/inducer T lymphocyte (Th/i) is in close membrane-to-membrane contact with a macrophage (Mac) (arrowhead) in the interstitial space. MC, myocardial cell. No staining with uranyl acetate or lead citrate. Scale, 1 μ m.

T4/T8 ratios lower than 1.0.

(2) Immuno-electron Microscopic Observations in the Myocardial Biopsies

T lymphocytes were identifiable at the electron microscopic level. These T cells showed positive reaction products focally along the surface of their cell membrane and/or in the cytoplasm. Occasionally, the reaction product was uniformly distributed along the entire cell membrane surface. The T cells usually had a high nuclear-cytoplasmic ratio.

In DCM patients both Leu 2a⁺ and Leu 3⁺ lymphocytes were identifiable in the myocardium. Some Leu 2a⁺ T lymphocytes were present in and around the fibrotic foci (Fig. 3). Leu 3⁺ T cells were sometimes in close contact with other lymphocytes and macrophages.

In MYO patients both Leu 2a⁺ and Leu 3⁺ lymphocytes were often present in and around myocardial foci. Myocardial biopsies obtained from patient 1 showed many Leu 3⁺ lymphocytes, some of which were in close contact with macrophages (Fig. 4).

DISCUSSION

The histologic hallmark of myocarditis is an inflammatory infiltrate of mononuclear cells in the vicinity of damaged myocardial cells. These cellular elements are believed to play a crucial immunologic role in the pathogenesis of the disease. Recently several authors have described infiltrations of lymphocytes in endomyocardial biopsies from some patients with DCM^{5,7-9}. Much attention has been focused on the immunologic role of lymphocytes, including T cells, in the pathogenesis of the myocardial lesions in DCM and myocarditis^{2-6,8,9}. However, there have been only a few in situ analysis of lymphocyte subsets in the myocardial lesions of patients with DCM or myocarditis, and these studies were restricted to light microscopic immunohistochemistry.

T lymphocytes are divided into several subsets, which have different immunological functions in vivo. These T cell subsets can be identified by lymphocyte surface markers: Leu 2a⁺ (OKT8⁺) lymphocytes are cytotoxic/suppressor T cells and Leu 3⁺ (OKT4⁺) lymphocytes are helper/

inducer T cells. To analyze the lymphocyte subsets in situ in myocardial lesions; we examined myocardial biopsies obtained from 11 DCM patients and three MYO patients, using a battery of monoclonal antibodies and a sensitive immunohistochemical staining technique. In 9 of the 11 DCM patients Leu 2a⁺ cytotoxic/suppressor T cells were more numerous than Leu 3⁺ helper/inducer cells (Table II), and the mean T4/T8 ratio was 0.74 ± 0.63 (mean $\pm$ SD). Recently, Linder et al. used immunohistochemical technique to examine non-inflamed post-mortem hearts and reported a T4/T8 ratio of 1.44.¹³ In contrast, a decreased T4/T8 ratio was found in the myocardium of most of our DCM patients. This low T4/T8 ratio in the myocardium in situ may reflect some immunologic change in myocardial lesion. The larger number of Leu 2a⁺ cells than of Leu 3⁺ cells in the myocardium of most DCM patients may reflect a rise in cytotoxic T cells. It is already known that cytotoxic T cells damage target cells either by lymphokine production or by a direct cytotoxic effect.¹⁵ To determine whether cytotoxic T lymphocytes are the main cause of the destruction of cardiocytes in the myocardial lesions of DCM. Many more biopsies from patients with DCM must be examined immunohistochemically.

Only three MYO patients were examined, and their T4/T8 ratios in the myocardium were very different (0.11, 0.77 and 2.53). No significant elevation occurred in the titers of neutralizing antibodies to enteroviruses, they had clinical features suggestive of viral infection, such as flu-like and gastrointestinal symptoms, preceding their cardiac symptoms. We have noted that some T cells are in close contact with cardiocytes in the myocardium of mice with experimental coxsackie B3 virus myocarditis.¹⁰ These T lymphocytes appear to be effector cells involved in cardiocyte destruction in vivo.¹⁰ In conventional electron microscopic studies of endomyocardial biopsies from myocarditis patients, we have occasionally noted membrane-to-membrane contact between lymphocytes and damaged cardiocytes.¹⁴ In the present study Leu 2a⁺ cytotoxic/suppressor T cells were often seen in the myocardial lesions of MYO patients. We were not able to find membrane-to-membrane contact between Leu 2a⁺ cells and cardiocytes in the present study, perhaps because the sections were too thin.

In the MYO patients Leu 3⁺ helper/inducer T cells were often present in and around inflamma-

tory foci. Immunoelectron microscopy showed that some Leu 3⁺ cells were in close contact with macrophages. Contacts between lymphocytes and macrophages have also been observed in the myocardium of mice with coxsackie B3 virus myocarditis.^{13,14} It is already known that helper T cells are maximally stimulated when they are in direct contact with macrophages. The activation of helper T cells may stimulate B cells and cytotoxic T cells.¹⁵ The fact that there is direct contact between macrophages and lymphocytes in the myocardium of MYO patients suggests that the activation of helper T cell is commonly involved in disease processes.

In conclusion, myocardial biopsies demonstrated predominant infiltrations of cytotoxic/suppressor T cells, and T4/T8 ratios were less than 1.0 in most cases of DCM. Our results support the possibility that cytotoxic/suppressor T cells play some crucial role in the development of myocardial lesions in DCM. Our three MYO patients had T4/T8 ratios which varied widely and differed greatly from Linder's findings in non-inflamed hearts¹³ and ours in DCM hearts. It is possible that these wide variations in T4/T8 ratios may be related to the stage of myocarditis, or to differences in sampling and size of biopsies. Further evaluations are needed to determine whether common cell-mediated immune mechanisms are involved in the pathogenesis of these myocardial diseases.

Acknowledgements

The authors wish to thank Dr. Gotatro Yamada of the First Department of Internal Medicine, Okayama University for aid in the immunohistochemical study. We are grateful to Dr. Alice Cary for correction of the manuscript, and Mrs. Akiyo Saito, Miss Keiko Uezono and Mr. Sumie Fuka for their technical assistance.

REFERENCES

1. HURST JW: The Heart, Arteries and Veins. McGraw-Hill, New York, 5th ed, pp 1278-1299
2. FOWLES RE, BIEBER CP, STINSON EB: Defective in vitro suppressor cell function in idiopathic congestive cardiomyopathy. *Circulation* 59: 483, 1979
3. ECKSTEIN R, MENPEL W, BOLTE H-D: Reduced suppressor cell activity in congestive cardiomyopathy and in myocarditis. *Circulation* 65: 1224, 1982
4. DAS SK, CASSIDY JT, HUBER SA: Immunity in dilated cardiomyopathy. *Heart and Vessel* 1 (Supple 1): 205, 1985
5. ZEE-CHENG C, TSAI CC, PALMER DC, CODD JE, PENNINGTON DG, WILLIAMS GA: High

- incidence of myocarditis by endomyocardial biopsy in patients with idiopathic congestive cardiomyopathy. *J Am Coll Cardiol* 3: 63, 1984
6. WOODRUFF JF: Viral myocarditis: A review. *Am J Pathol* 101: 426, 1980
 7. MASON JW, BILLINGHAM ME, RICCI DR: Treatment of acute inflammatory myocarditis assisted by endomyocardial biopsy. *Am J Cardiol* 45: 1037, 1980
 8. EDWARDS WD, HOLMES DR Jr, REEDER GS: Diagnosis of active lymphocytic myocarditis by endomyocardial biopsy: Quantitative criteria for light microscopy. *Mayo Clin Proc* 57: 419, 1982
 9. DEC GW Jr, PALACIOUS IF, FALLON JT, ARETZ HT, MILLS J, LEE DC-S, JOHNSON RA: Active myocarditis in the spectrum of acute dilated cardiomyopathies. *N Eng J Med* 312: 885, 1985
 10. DEGUCHI H, KITAURA Y, MORITA H, KOTAKA M, KAWAMURA K: Cell-mediated immunity in coxsackie B3 virus myocarditis in mice – in situ characterization by monoclonal antibody of mononuclear cell infiltrates. *Heart and Vessel* 1 (Supple 1): 221, 1985
 11. DEGUCHI H, KITAURA Y, HAYASHI T, KOTAKA M, MORITA H, KAWAMURA K: Immunohistochemical study of the myocardium in murine coxsackie B3 virus myocarditis using monoclonal antibodies – Significance of Lyt 1 antigen-bearing lymphocytes in cell-mediated immunity—. *Jpn Circ J* 50: 1268, 1986
 12. McLEAN IW, NAKANE PK: Periodate-lysine-Paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J Histochem Cytochem* 22: 1077, 1974
 13. LINDER J, CASSLING RS, ROGLER WC, WILSON JE, MARKIN RS, SEARS TD, McMANUS BM: Immunohistochemical characterization of lymphocytes in uninflamed ventricular myocardium. *Arch Pathol Lab Med* 109: 917, 1985
 14. KITAURA Y, DEGUCHI H, KOTAKA M, MORITA H: Cardiomyopathy and myocarditis. Clinical and experimental follow-up study of viral myocarditis. *Jpn Circ J* 1987 (in press)
 15. HERSCOWITZ HB: Immunophysiology: Cell function and cellular interactions in antibody formation. In *Immunology III* ed by BELLANTI JA, WB Saunders Company, Philadelphia, London, Toronto, Tokyo, 1985 pp 117–159