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Natural Killer Cells in Dilated Cardiomyopathy

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YOKOYAMA, A. *Natural Killer Cells in Dilated Cardiomyopathy.* Tohoku J. exp. Med., 1988, **154** (4), 335-344 —— Dilated cardiomyopathy is associated with various immunological abnormalities, such as decreases in the activity and subsets of suppressor T cells and in the activity of natural killer cells, suggesting the involvement of immunological mechanisms in the pathogenesis of this disease. I assayed several subsets and the activity of natural killer cells in the peripheral blood of 44 patients with dilted cardiomyopathy and compared the results with the clinical course. Compared with normal individuals, the patient group showed increases in cells positive for the subsets Leu 7 and Leu 11. Double staining method revealed that natural killer cell groups were positive for Leu 7, Leu 11 and Leu 15 but negative for Leu 2a. The activity of natural killer cells was decreased in all cases, particularly in the subgroup with mild illness. Addition of interleukin 2 (IL-2) caused no increase in activity in any of the cases. Compared with the mild subgroup, the subgroup with severe illness included more male patients and had a shorter clinical course ($p < 0.05$). Of the 43 patients who underwent gallium 67 (Ga-67) myocardial scintigraphy, four demonstrated an accumulation of the contrast material in the myocardium, and all of whom belonged to the mild subgroup. These results suggest that inhibition of the function or activation of natural killer cells and the pathophysiology of chronic myocarditis are intimately associated with the occurrence of at least the mild form of dilated cardiomyopathy. ———— dilated cardiomyopathy; lymphocyte subset; natural killer cell; gallium 67 myocardial scinitigraphy

Dilated cardiomyopathy is a disease that carries a poor prognosis and for which no radical therapy is available (Report of the WHO 1980; Fuster et al. 1981). Its aetiology is unknown at present, but various factors are thought to be involved. Immunological abnormalities reported in association with dilated cardiomyopathy include decreases in the activity of suppressor T cells (Reinhold et al. 1982), cells positive for OKT8 (Yokoyama et al. 1985a, b), and natural killer cells (Anderson et al. 1982). Attention has also been focused on the association of dilated cardiomyopathy with chronic myocarditis, and lymphocyte infiltration in the myocardium of patients with dilated cardiomyopathy has been reported

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(O'Connell et al. 1981, 1984; Hammond and Anderson 1983; McManus et al. 1984; Yokoyama et al. 1986).

The author assayed several subsets and the activity of natural killer cells in the peripheral blood from 44 patients with dilated cardiomyopathy and compared the results with their clinical condition. In addition, gallium-67 (Ga-67) myocardial scintigraphy was performed in 43 patients to investigate the possible relationship of dilated cardiomyopathy to chronic myocarditis.

Subjects and Methods

Subjects

The 44 subjects consisted of 35 males and 9 females who were diagnosed as having dilated cardiomyopathy on the basis of the Idiopathic Cardiomyopathy Research Group Guide for Diagnosis of Idiopathic Cardiomyopathy by the Ministry of Health and Welfare (Idiopathic Cardiomyopathy Research Group 1983). The mean age of these patients was 54 years. Table 1 shows the parameters of cardiac function obtained at examination. The 44 patients were divided into two subgroups, a mild subgroup with a left ventricular end diastolic dimension of 56–61 mm, and a severe subgroup with a left ventricular end-diastolic dimension of 62 mm or more. Controls were 80 healthy volunteers matched in age and sex.

Methods

Assay of lymphocyte subsets

Monoclonal antibodies were added to 100 ml of ethylene diamine tetraacetic acid-treated peripheral blood and incubated at 4°C for 30 min. The reaction mixture was hemolyzed in an ammonium chloride solution. The lymphocytes were stained by the direct fluorescent antibody technique, and the number of cells positive for the fluorescence was determined by laser flow cytometer (Spectrum III, Ortho Co., Raritan, NJ, USA). For the assay of Leu 11 by the direct fluorescent antibody technique, specimens were rinsed and then stained with a combination of FITC-labeled Leu 7 and phycoerythrin-labeled anti-Leu 2a,

Table 1. *Patients with dilated cardiomyopathy*

Number of patients	44
Age (years)	54±10
Sex (M • F)	35 • 9
Duration of symptoms (months)	40±22
Functional class (NYHA*)	
Number of grade II	24
Number of grade III	14
Number of grade IV	6
Left ventricular ejection fraction (%)	33±11
Pulmonary artery wedge pressure (mmHg)	13±7
Left ventricular end diastolic dimension (mm)	62±6
Number of mild subgroup (56~61 mm)	24
Number of severe subgroup (62~82 mm)	20

Values are means±S.D.

* NYHA, New York Heart Association.

anti-Leu 11 or anti-Leu 15. The number of positive cells in the lymphocyte fraction was determined by a FACS-420 laser flow cytometer (Becton-Dickinson Co., Sunnyvale, CA, USA) (Yokoyama et al. 1985a, b). Double staining was carried out in 10 cases (10/44, 23%) who showed marked increase in cells positive for Leu 7 and Leu 11.

Natural killer cell activities before and after the addition of IL-2 (IL-2)

K-562 cells, a human erythroleukemic cell line, maintained in RPMI 1640 containing 10% fetal calf serum were used as the targets for natural killer cells. Target cells were radiolabeled by incubating with 200 μCi chronium-51 (Cr-51) (Daiichi Radioisotope Inst., Tokyo) for 1 hr at 37°C. The cells were then washed 4 times and adjusted to a concentration of 2×10^5 cells/ml. Mononuclear cells for use as effector cells isolated from the heparinized peripheral blood by densitometric centrifugation with Ficoll-Hypaque (specific gravity, 1,077 ; $400\times g$; 30 min). Effector cells adjusted to a concentration of 4×10^6 cells/ml. Culture solution were composed of 40% human serum from blood type AB or a mixture of 40% human serum from blood type AB and 10% IL-2. Labeled target cells, effector cells and culture solution, 50, 100 and 50 μl were coincubated quadruplicately in round-bottom microtiter plates (Nunc, Denmark) such that the effector : Target ratio was 40 : 1. The final volume in each well was 200 μl. The plates were incubated for 4 hr at 37°C in 5% CO_2. One hundred fifty microliters of supernatant was removed and counted in a gamma counter. Spontaneous release was assessed by a series of wells containing no effector cells. Maximum release was obtained by target cells treated by 1 M HCl. Natual killer cell activity (%) was calculated by the following formula (Herberman and Callewaert 1985 ; Tanaka et al. 1985) :

Natural killer cell activity (%) = (released Cr-51 CPM-spontaneously released Cr-51 CMP)/(maximum released Cr-51 CPM-spontaneously released Cr-51 CPM) $\times$ 100.

Myocardial scintigraphy using gallium-67

Single Photon Emission Computed Tomography (SPECT) images were photographed using a Shimadzu PHO/GAMMA LFOV equipped with a middle-energy collimator and a Siemens ZLC/75 rotor camera 72 hr after an intravenous injection of ^{67}Ga-citrate at a dose of 3.0 mCi/3.0 ml. Three photo peaks of Ga-67 were used : 93, 184 and 296 Kev. The window width was 20%. SPECT data were collected using a DEC PDP11/34 (GAMMER-11) at a sampling angle of 10 with a slice interval of 18 mm over a sampling time of 60 sec. Image reconstruction was carried out by the FPB method with a Sheep & Logan filter (Yokoyama et al. 1986).

Statistical analysis

Values were given in terms of mean $\pm$S.D. The unpaired t-test was used for statistical analysis.

Results

Surface characteristics

Analysis of lymphocyte subsets

Fig. 1 shows the data for the lymphocyte subsets in the dilated cardiomyopathy and control groups. The levels of Leu 7 and Leu 11, surface markers of natural killer cells, in the dilated cardiomyopathy group were significantly higher than those of the control group : 28 ± 11 and 22 ± 11, respectively ($p<0.01$, $p<0.01$). The dilated cardiomyopathy group showed a higher level (43 ± 10) of T4, a surface maker of helper/inducer T cells, than the control group (40 ± 6 ; $p<0.05$). However, the dilated cardiomyopathy group exhibited a significantly level (24 ± 8) of T8, a surface marker of suppresson T cells, than the control group (27 ± 5 ; $p<0.05$). Therefore, the T4/8 ratio of the dilated car-

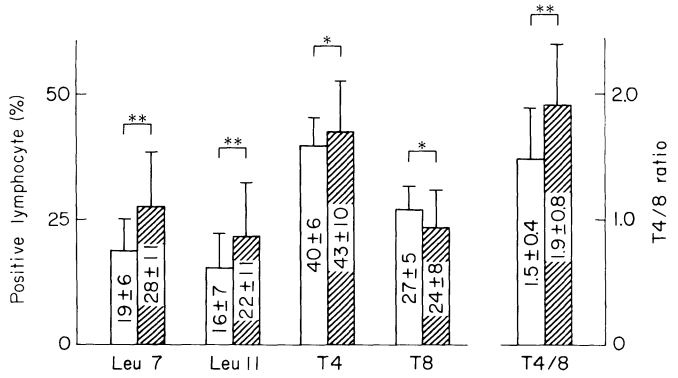

Fig. 1. Analysis of lymphocyte subset.
Values are means±S.D. $*p<0.05$, $**p<0.01$ against controls.
□, control; ▨, dilated cardiomyopathy.

diomyopathy group (1.9±0.8) was significantly higher than that of the control group (1.5±0.4; $p<0.01$).

Analysis by double staining

Leu 7 and Leu 11 were assayed by double staining in the 10 cases (23%) who showed an increase in these subsets in the first assay. The results for one of these

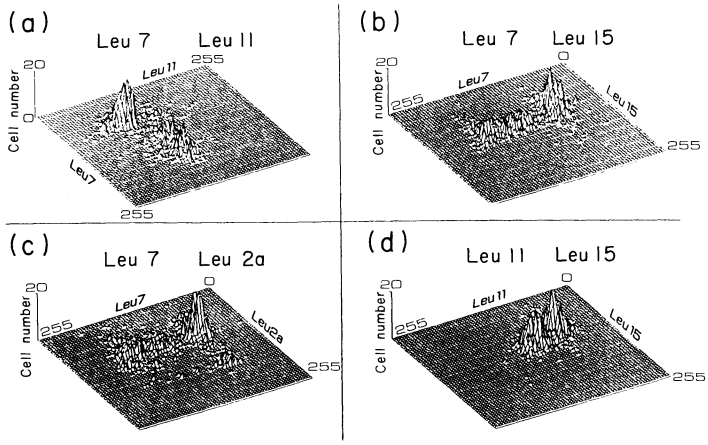

Fig. 2. Distribution of double staining-positive lymphocytes determined with a FACS 420 laser flow cytometer.
(a) Most of the cells stained with a combination of Leu 7 and Leu 11 were simulatineously positive for both subsets. (b) Most of the cells stained with a combination of Leu 7 and Leu 15 were simultaneously positive for both subsets. (c) Most of the cells stained with a combination of Leu 7 and Leu 2a were positive for Leu 7 but negative for Leu 2a. (d) Most of cells stained with a combination of Leu 11 and Leu 15 were simultaneously positive of both subsets.

cases are shown in Fig. 2. The overall results for the 10 cases showed the following tendencies:

(a) Most of the cells stained with a combination of Leu 7 and Leu 11 were simultaneously positive for both subsets.

(b) Most of the cells stained with a combination of Leu 7 and Leu 15 were simultaneously positive for both subsets.

(c) Most of the cells stained with a combination of Leu 7 and Leu 2a were positive for the former and negative for the latter.

(d) Most of the cells stained with a combination of Leu 11 and Leu 15 were simultaneously positive for both subsets.

Double staining revealed that a majority of the cells were positive for Leu 7, Leu 11 and Leu 15 and negative for Leu 2a in the dilated cardiomyopathy group.

Natural killer cell activities in the presence or absence of 10% IL-2

Fig. 3 shows the results for the entire dilated cardiomyopathy group and the control group as well as for the mild and severe dilated cardiomyopathy subgroups. Compared with the control group ($44\pm6\%$), the entire dilated cardiomyopathy group showed significantly lower natural killer cell activity ($35\pm6\%$; $p<0.01$). The mild dilated cardiomyopathy subgroup (mild dilation) showed markedly lower activity, $16\pm7\%$ ($p<0.01$). The natural killer cell activity in the severe dilated cardiomyopathy subgroup (sever dilation) was $46\pm6\%$, showing no significant difference from the control group.

With the addition of 10% IL-2, the natural killer cell activity in the entire group was $37\pm8\%$, significantly lower than that of the control group ($48\pm6\%$; $p<0.01$). The mild subgroup had markedly lower natural killer cell activity,

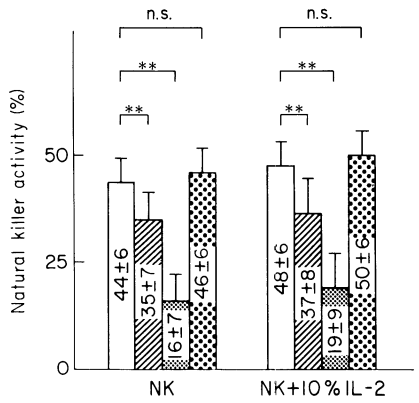

Fig. 3. Natural killer activity.
NK, natural killer activity; IL-2, interleukin 2.
□, control ($n=40$); ▨, dilated cardiomyopathy ($n=38$); ▩, mild dilatation ($n=20$); ▩, severe dilation ($n=18$).
Values are means±S.D. $^{**}p<0.01$; n.s., not significant.

TABLE 2. *Patients with dilated cardiomyopathy ; mild dilatation and severe dilation*

	Mild	Severe
Number of patient		
Male	17	18
Female	7	2
Age (years)	55±5	50±6
Duration of symptons (months)	44±9	37±8*

Values are means±S.D.
* $p<0.05$ agaist values of mild dilatation.

19±9% ($p<0.01$), whereas the severe subgroup (50±6%) was not significantly different from the control group.

Clinical characteristics of the severe and mild subgroups

Table 2 shows the clinical characteristics including age, clinical course and sex distribution in the severe and mild subgroups. The mean ages of the mild and severe subgroups were 55±5 years and 50±6 years, respectively. The severe subgroup was thus slightly, but not significantly, younger. The duration from onset in the mild subgroup was 44±9 months. In the severe subgroup, it was 37± 8 months. Thus, in the mild cases the clinical course was longer ($p<0.05$). While the 24 mild patients consisted of 17 males and 7 females, an M/F ratio of 2.4 : 1, the severe subgroup consisted predominantly of males (18 males and 2 females), with an M/F ratio of 9 : 1.

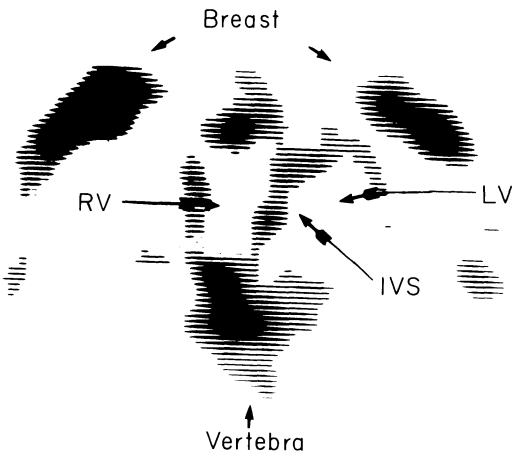

Fig. 4. Gallium-67 myocardial scintigraphy.
Diffuse accumulation was found in the myocardium.
LV, left ventricle ; RV, right ventricle ; IVS, interventricular septum.

Ga-67 myocardial scintigraphy

Planar images were photographed in all 44 patients with dilated cardiomyopathy. Ga-67 scintigraphy revealed no myocardial accumulation of the contrast material in any of the cases.

Ga-67 accumuated in the myocardium in 4 of the 43 patients in whom SPECT photos were taken. In the SPECT image, one patient showed grade II findings, with diffuse accumulation in the myocardium which was less marked than that in the spine (Yokoyama et al. 1986). The results in this case are shown in Fig. 4. Three patients showed grade I findings, with local accumulation in the myocardium. All of these 4 patients were from the mild subgroup. Of the 30 controls with chest diseases other than heart disease, none exhibited myocardial accumulation.

Thus, accumulation of Ga-67 in the myocardium was observed in 4 (9%) of the 44 patients with dilated cardiomyopathy ; all 4 were from the mild subgroup.

Discussion

The data on lymphocyte subsets indicated that the patients with dilated cardiomyopathy, had an overall decrease in OKT8, compared with the control group. Cells positive for OKT8 are considered to include suppressor and cytoxic T cells. However, since there was no decrease in the cells positive for Leu 7, which is believed to be present in cytotoxic T cells, natural killer cells and killer cells, a decrease in suppressor T cells was probably responsible for the decrease in cells positive for OKT8 (Yokoyama et al. 1985a, b). This conclusion is consistent with the results of Reinhold et al. (1982), who observed decreased activity of suppressor T cells in the peripheral blood of patients with dilated cardiomyopathy and suggests that enhanced humoral immunity is involved in this disease. This idea is supported by the presence of myocardial antibodies detected by Maish et al. (1983) in the sera of patients with dilated cardiomyopathy. On the other hand, the large number of cells positive for Leu 7 and Leu 11 indicated increases in cytotoxic T cells, natural killer cells and killer cells (Yokoyama et al. 1985a, b). Double staining in the present study revealed that the majority of cells were positive for Leu 7, Leu 11 and Leu 15 and negative for Leu 2a. In this regard, (Abo and Balch 1981 ; Abo et al. 1984) reported that there were some natural killer cells with weak activity. Kusukawa (1986) has indicated the reduction of natural killer cell activity in dilated cardiomyopathy patients whose cardiac function were extremely low. Yokoyama et al. (1985c) have indicated the disorder of IL-2 receptor in dilated cardiomyopathy patients. Anderson et al. (1985) have indicated the natural killer cell activity in this disease was frequently deficient although Leu 7 and Leu 11 were in normal range. They also have indicated interferon was a well-difined inducer of natural killer activity. In my study, a decrease in natural killer activity was found in all patients with dilated

cardiomyopathy, particularly in the mild subgroup, whereas the severe subgroup showed no difference from the control. Furthermore, IL-2 did not enhance the cell activity, indicating that the natural killer cells were, in fact, pre-natural killer cells, and that the function or activation of the natural killer cells was inhibited. Some of the patients with dilated cardiomyopathy were probably refractory to IL-2. The possible association of dilated cardiomyopathy with viral myocarditis is gaining attention, and its refractoriness to IL-2 is of interest because natural killer cells play an important role in viral infection.

Ito et al. (1971) have suggested that accumulation of Ga-citrate is caused by the vasodilation, vascular hypertrophy and increase in vascular permeability due to tumour or inflammation. Histologically, this contrast material is believed to accumulate in phlogocytes such as lymphocytes, leukocytes and histiocytes. The possible association of dilated cardiomyopathy with myocarditis suggests that chronic inflammation is responsible for the accumulation of ^{67}Ga-citrate in the heart of patients with dilated cardiomyopathy (O'Connell et al. 1981, 1984; Yokoyama et al. 1986). However, there was no biochemical evidence of inflammation, and a serum virological study for coxsackie virus B (types 1-6) revealed no increase in the antibody titres. Moreover, biopsy of the myocardium in the right ventricular septum (Kurotaki 1972) revealed no histopathologic evidence of inflammation in cases showing ^{67}Ga accumulation in the myocardium. O'Connell es al. (1984) have indicated the difficulty of judging the presence or absence of inflammation of the entire heart except by biopsy of the right ventricular septum. However, patients with Ga-67 accumulation in the myocardium were all from the mild subgroup. This is interesting in view of the report that the administration of immunosuppressive agents, including prednisolone, resulted in improvement in the cardiac function of some patients with dilated cardiomyopathy who showed myocardial Ga-67 accumulation (O'Connell et al. 1981).

Various factors seem to be involved in the aetiology of dilated cardiomyopathy. The present study revealed increases in the number of natural killer cells positive for Leu 7 and Leu 11, surface markers of the natural killer cell. However, the activity of natural killer cells was decreased, and IL-2 failed to enhance this activity. Most of the patients who showed marked inhibition of natural killer cell function or activation were in the mild subgroup, as were all the patients exhibiting Ga-67 accumulation in the myocardium. These findings suggest that inhibition of natural killer cell function or activation and the presence of chronic myocarditis are the possible causes of heterogeneous dilated cardiomyopathy. I hope the progress of natural killer cell study and gallium 67 myocardial scintigraphic study will make possible not only the elucidation of pathogenesis and diagnosis of dilated cardiomyopathy and chronic myocarditis but also immuno suppressive agents, including prednisolone, and immuno activative agents, including interferon, might be the first step on the new agents

of dilated cardiomyopathy.

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