Immunologic Identification of Lymphocyte Subsets in Experimental Murine Myocarditis With Encephalomyocarditis Virus

Different Kinetics of Lymphocyte Subsets Between the Heart and the Peripheral Blood, and Significance of Thy 1.2+ (Pan T) and Lyt 1+, 23+ (Immature T) Subsets in the Development of Myocarditis

Chiharu Kishimoto, Kagemasa Kuribayashi, Kanzo Fukuma, Tohru Masuda, Nobuyoshi Tomioka, Walter H. Abelmann, and Chuichi Kawai

To clarify the immune mechanism in myocarditis, immunofluorescence techniques with laser flow cytometry were used to examine serial changes in lymphocyte subsets in the heart, spleen, and peripheral blood of DBA/2 and BALB/c mice inoculated with encephalomyocarditis virus (Experiment I). B cells were identified by staining with fluorescein isothiocyanate-labelled rabbit anti-mouse immunoglobulin. T-cell subsets were identified with rat anti-Thy 1.2, and nonpolymorphic Lyt 1 and Lyt 2 monoclonal antibodies plus fluorescein isothiocyanate-labelled anti-mouse immunoglobulin. On days 7 and 14 postinfection, the percentage of Thy 1.2+ (pan T) cells in both strains had decreased in the peripheral blood; B cells showed no significant changes throughout the entire period. On the other hand, Thy 1.2+ (pan T) and Lyt 1+, 23+ (precursor and immature) T cells appeared to occupy the major portion of the myocardium on days 7 and 14 when congestive heart failure developed. To confirm this, serial immunohistologic studies (immunoperoxidase staining) of the hearts of DBA/2 and BALB/c mice with encephalomyocarditis virus-induced myocarditis were performed (Experiment II). In Experiment II, most of the stained cells in the hearts of both strains were Thy 1.2 positive and Lyt 1 and Lyt 2 positive on days 7 and 14. Thus, Experiments I and II demonstrated that lymphocytes at the site of inflammation in acute viral myocarditis carried antigenic markers that differed from those of peripheral lymphocytes and suggested that Thy 1.2+ (pan T) cells, especially the Lyt 1+, 23+ subset (immature T cells and T-cell subset precursors) were involved in the development of myocarditis in these animals. (Circulation Research 1987;61:715-725)

The importance of immune dysfunction in myocarditis and idiopathic dilated (congestive) cardiomyopathy has been postulated but remains to be demonstrated. In recent years, much work, based on the virus-immune hypothesis, has been done using experimental animal models on the pathogenesis of cardiomyopathy. According to the data obtained, viral myocarditis initiates a disease process that is then perpetuated by abnormal immune responses.

Recently, we have identified congestive heart failure during the acute stage of encephalomyocarditis virus infection in BALB/c mice and severe myocarditis in similarly infected DBA/2 mice. It has already been reported that the cardiac lesions of DBA/2 mice with chronic myocarditis are similar to those seen in some patients with dilated cardiomyopathy. In addition, we found that lymphocytes were involved in the severity and development of myocarditis in these two experimental animal models. However, serial changes in T-lymphocyte subsets in experimental viral myocarditis have not been investigated.

In this study, an immunofluorescence method with laser flow cytometry was used to examine serial changes in T-lymphocyte subsets in the hearts, spleens, and peripheral blood of DBA/2 and BALB/c mice inoculated with encephalomyocarditis virus (Experiment I). Parallel studies were performed in which serial immunohistologic changes in the hearts of DBA/2 and BALB/c mice with encephalomyocarditis virus-induced myocarditis were investigated (Experiment
II). The possible role of infiltrating T-cell subsets in the development of virus-induced myocarditis is discussed.

Materials and Methods

Experimental Infections

The myocardiotrophic variant of encephalomyocarditis virus was used; the virus stock was prepared in cultures of human amnion cells in Eagle’s minimum essential medium. Cell-conditioned medium containing suspensions of the virus was centrifuged after the cytopathic effect had developed. Virus stocks had a titer of $10^{5.5}$ TCD50 (50% tissue culture infective dose) per 0.1 ml determined in tissue cultures of human amnion cells and were stored at $-70^\circ$ C until use.

Experiment I

The precise experimental infection methodology has been described previously.1,19 In brief, inbred DBA/2 ($n = 378$) and BALB/c ($n = 402$) mice, 5 weeks of age, were inoculated intraperitoneally with 0.1 ml virus suspension containing a $10^{3.5}$ TCD50. After confirming myocarditis from the gross appearance of the heart (yellowish-white patches on the surface, Figure 1), the heart, spleen, and peripheral blood were processed for serial determination of lymphocyte subsets. Five-week-old noninfected DBA/2 ($n = 24$) and BALB/c ($n = 24$) mice were used as controls.

Staining methods were similar to those described previously.1,19 Spleen and heart were minced gently using a sterile stainless steel mesh. Heart samples from 8 to 15 animals were pooled to obtain adequate numbers of lymphocytes. After mincing, the cell suspension was rapidly pipetted with a sterile Pasteur pipette into 20–25 ml Hanks’ balanced salt solution, filtered through cotton wool to eliminate debris, and centrifuged at 1,500 rpm for 5 minutes. The cells were then washed twice with Hanks’ balanced salt solution. Heparinized peripheral blood (1.2–1.5 ml) was washed twice with Hanks’ balanced salt solution, and the red cells were then lysed by hypotonic shock. Lymphocytes were obtained from the heart, spleen, and peripheral blood by Ficoll-Metrizoate gradient centrifugation, suspensions being layered carefully over 4 ml of Ficoll-Metrizoate and centrifuged at 2,000 rpm for 15 minutes. The lymphocytes thus obtained were treated with 0.2% trypan blue and then counted in a standard hemocytometer; cell viability was found to be at least 95%. The cells were finally suspended at a concentration of $5 \times 10^9 - 1 \times 10^8$ cells/ml in RPMI-1640 medium with 2.5% fetal calf serum and 0.1% NaN3.

B cells were determined by staining with fluorescein isothiocyanate-labelled rabbit anti-mouse immunoglobulin antibody (Miles-Yeda, Naperville, Ill.). T cells were stained with monoclonal rat anti-Thy 1.2 (HO-13.4) antibody against pan T lymphocytes, monoclonal rat anti-Lyt 1, rat anti-Lyt 2, and rat anti-Lyt 1 plus rat anti-Lyt 2 antibodies as the primary antibodies,2 followed by fluorescein isothiocyanate-labelled rabbit anti-mouse immunoglobulin antibody as the secondary. Rat monoclonal antibodies were obtained as culture supernatants of the respective hybridoma cells originally supplied by the Cell Distribution Center, Salk Institute, San Diego, Calif.

Fluorescein isothiocyanate-labelled anti-mouse immunoglobulin antibody was diluted 15 times, and rat monoclonal antibodies 5 times. The cells (1 $\times 10^9 - 1 \times 10^8$ in 0.1 ml RPMI-1640 medium with 2.5% fetal calf serum and 0.1% NaN3) were put into V-bottomed wells of a microtiter plate (Limbro Scientific, Hamden, Conn.) and centrifuged at 1,500 rpm for 3 minutes. After centrifugation, the cell pellet was suspended in either 100 $\mu$l RPMI-1640 medium with 2.5% fetal calf serum and 0.1% NaN3 or the primary antibodies. After incubation for 30 minutes at 4°C, the cells were washed 3 times and suspended in 100 $\mu$l of the secondary antibody. The cells were washed twice with RPMI-1640 medium containing 2.5% fetal calf serum and 0.1% NaN3, after 30 minutes incubation at 4°C with the secondary antibody and then resuspended in 1 ml of the same medium.

The preparations were immediately analyzed by laser flow cytometry (Ortho Spectrum III interfaced to digital electronics, Ortho Diagnostic System K.K., Westwood, Mass.); the fraction of mononuclear cells binding a given antiserum was determined by comparing the number of fluorescing cells with the total cell population by forward light scatter. Approximately $5 \times 10^7 - 5 \times 10^6$ cells/heart sample and $5 \times 10^6$ cells/spleen and peripheral blood samples were analyzed. The analysis was performed after determining the area occupied by lymphocytes on the cytogram (Figure 2).

The percentages of T cells and T-cell subsets were obtained by subtracting the percentage of B cells from the total mononuclear cell population.

Figure 1. Gross pathology of the heart of DBA/2 mice on day 45. After day 7, yellowish-white patches (arrows) are seen on the surface of the ventricles. Scale = 1 mm.
those obtained after staining with each monoclonal rat antibody and fluorescein isothiocyanate-labelled rabbit-anti-mouse immunoglobulin antibody.

Based on the assumptions that all T cells were positive for Thy 1.2 and bore either Lyt 1, Lyt 2, or both, calculations were as follows:

\[(\text{Lyt } 1^+, 23^-) + (\text{Lyt } 1^+, 23^-) + (\text{Lyt } 1^-, 23^+) = \text{Thy 1.2 positive or Lyt } 1^+ \text{ Lyt } 2^+ \]  
\[(\text{Lyt } 1^+, 23^-) + (\text{Lyt } 1^-, 23^-) = \text{Lyt 1 positive} \]  
\[(\text{Lyt } 1^-, 23^+) + (\text{Lyt } 1^-, 23^-) = \text{Lyt 2 positive} \]

where asterisk indicates that a higher value was cited.

Interpretations are as follows: Thy 1.2+ = pan T cells; Lyt 1+, 23+ = precursors of T-cell subsets and immature T cells; Lyt 1+, 23- = helper/inducer T cells; and Lyt 1-, 23+ = suppressor/cytotoxic T cells.

**Experiment I**

Inbred DBA/2 (n = 92) and BALB/c (n = 98), 5 weeks of age, were inoculated intraperitoneally with 0.1 ml virus suspension containing 10^3 TCD50. After confirming myocarditis (Figure 1), hearts from both strains were sectioned on days 7, 14, and 30–45 postinoculation along the long axis through both the atria and ventricles, one half being then processed for a virologic study and the other half for an immunohistologic study. Five-week-old noninfected DBA/2 (n = 2) and BALB/c (n = 2) mice were prepared as controls.

Four micron-thick sections were cut from frozen blocks on a cryostat at −20°C, placed on glass slides, air-dried for 1 hour, and fixed in 95% cold methanol. Cell surface markers were demonstrated in situ by 3,3′-diaminobenzidine tetrahydrochloride immunoperoxidase staining, using the same series of monoclonal rat anti-mouse alloantigen antibodies as in Experiment I as the primary antibodies, and peroxidase-conjugated affinity column purified mouse anti-rat immunoglobulin antibody as the secondary. In addition, infiltrating B cells were stained by rat monoclonal antibody, Bet 1, which reacts specifically with the mouse immunoglobulin \( \mu \) allotypic marker of immunoglobulin h-6′ mouse strains, including both BALB/c and DBA/2. Incubation of cells with antibodies was performed in a moist chamber for 1 hour. The mouse anti-rat immunoglobulin antibody was raised in BALB/c mice by subcutaneous injection of rat immunoglobulin successively with complete and incomplete Freund’s adjuvant, and was partially purified by 50% ammonium sulfate precipitation, followed by affinity chromatography on a DEAE-cellulose column. Thereafter, the specific antibody was purified using a rat immunoglobulin-coupled Sepharose 4B affinity column, which could then be conjugated with horseradish peroxidase. Sections were counterstained with Mayer-hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific, Silver Spring, Md.) under glass coverslips. Control sections, treated with phosphate-buffered saline solution instead of the primary antibody, enabled the verification of both the absence of contaminating cross-reactions and the accuracy of the method.

For quantitation of lymphocyte subsets, the sections were examined by one examiner blindly: the number of lymphocytes stained in each section by a given monoclonal antibody was recorded with the total number of small round nucleated cells, and the percentage of stained lymphocyte was then calculated. This process was repeated for each lymphocyte focus, defined as an average of 50 or more lymphocytes. The lungs, liver, and other organs were also sectioned and stained with hematoxylin-eosin.

For virus isolation, hearts were ground with sea sand, and 1% suspensions in Eagle’s minimum essential medium were made. After centrifugation at 6,000 rpm for 30 minutes, 0.1 ml of each supernatant was inoculated into tube cultures of human amnion cells containing 1 ml Eagle’s minimum essential medium supplemented with 2% fetal calf serum. The tubes were examined for 7 days for the appearance of the characteristic cytopathic effect.

**Statistical Analysis**

Statistical analysis of the data was performed by an analysis of variance with multiple comparisons. A probability value of less than 0.05 was considered statistically significant. All results are expressed as mean ± SD.

**Results**

**Experiment I**

The incidence of myocarditis was 94.2% (356/378) in DBA/2 mice and 92.0% (370/402) in BALB/c mice. The survival rate observed on day 6 was 86.0% (325/378) in DBA/2 mice and 91.5% (368/402) in BALB/c mice. Tables 1 and 2 summarize the results of Experiment I. No lymphocytes were recovered from normal hearts by the Ficoll-Metrizoate gradient method.

As reported previously, in both strains the percentages of Thy 1.2+ (pan T) and Lyt 1+, 23+ (precursor) cells were decreased in the peripheral blood on days 7
Table 1. Serial Changes in Lymphocyte Subsets in the Peripheral Blood, Spleen, and Heart of DBA/2 Mice After Encephalomyocarditis Virus Inoculation

<table>
<thead>
<tr>
<th>Days</th>
<th>0 (n = 6)</th>
<th>7 (n = 6)</th>
<th>14 (n = 6)</th>
<th>30-45 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peripheral blood (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>28.0±6.2</td>
<td>27.8±13.8</td>
<td>30.3±12.3</td>
<td>21.6±6.5</td>
</tr>
<tr>
<td>Thy 1.2+</td>
<td>49.2±14.8</td>
<td>32.1±9.4*</td>
<td>20.6±4.2†</td>
<td>43.9±14.0</td>
</tr>
<tr>
<td>Lyt 1+, 23+</td>
<td>29.0±7.0</td>
<td>20.7±8.3</td>
<td>6.6±5.0‡</td>
<td>20.1±6.4</td>
</tr>
<tr>
<td>Lyt 1+, 23-</td>
<td>14.9±7.3</td>
<td>9.8±8.3</td>
<td>9.6±5.1</td>
<td>14.6±6.1</td>
</tr>
<tr>
<td>Lyt 1-, 23+</td>
<td>5.3±5.0</td>
<td>4.6±4.3</td>
<td>5.8±3.6</td>
<td>9.1±5.5</td>
</tr>
<tr>
<td>TLC (×10⁶)</td>
<td>2.5</td>
<td>2.3</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Spleen (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>51.1±4.1</td>
<td>45.2±9.5</td>
<td>45.5±11.4</td>
<td>53.5±6.7</td>
</tr>
<tr>
<td>Thy 1.2+</td>
<td>34.3±5.8</td>
<td>26.5±8.2</td>
<td>31.7±9.2</td>
<td>34.8±6.0</td>
</tr>
<tr>
<td>Lyt 1+, 23+</td>
<td>22.0±2.5</td>
<td>9.0±8.6*</td>
<td>17.7±9.4</td>
<td>23.3±7.3</td>
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<tr>
<td>Lyt 1+, 23-</td>
<td>9.4±3.8</td>
<td>9.6±6.5</td>
<td>7.8±3.0</td>
<td>6.0±2.3</td>
</tr>
<tr>
<td>Lyt 1-, 23+</td>
<td>4.0±3.7</td>
<td>5.9±5.6</td>
<td>6.6±5.9</td>
<td>5.4±3.8</td>
</tr>
<tr>
<td>TLC (×10⁶)</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Heart (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>14.3±9.4‡</td>
<td>9.6±6.2</td>
<td>29.7±3.9</td>
</tr>
<tr>
<td>Thy 1.2+</td>
<td>ND</td>
<td>75.1±13.4</td>
<td>78.0±12.7</td>
<td>49.8±27.1</td>
</tr>
<tr>
<td>Lyt 1+, 23+</td>
<td>ND</td>
<td>40.0±22.1</td>
<td>48.8±7.8</td>
<td>43.1±33.8</td>
</tr>
<tr>
<td>Lyt 1+, 23-</td>
<td>ND</td>
<td>11.6±13.3</td>
<td>19.0±10.1</td>
<td>4.4±7.1</td>
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<tr>
<td>Lyt 1-, 23+</td>
<td>ND</td>
<td>21.4±15.8</td>
<td>10.2±12.1</td>
<td>2.3±3.6</td>
</tr>
<tr>
<td>TLC (×10⁶)</td>
<td>3.3</td>
<td>4.3</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Values on day 0 were obtained from 5-week-old noninfected DBA/2 mice. Thy 1.2+ = pan T cell; Lyt 1+, 23+ = precursors of other T-cell subsets and immature T cells; Lyt 1+, 23- = helper/inducer T cells; Lyt 1-, 23+ = suppressor/cytotoxic T cells.

Abbreviations: ND, not detected; TLC, total lymphocyte counts per mouse (mean).

For heart tissue, each value was obtained from 8 to 15 pooled hearts (for adequate numbers of lymphocytes).

Values are mean±SD; *p<0.05, †p<0.01, ‡p<0.001 vs. control; §p<0.01 vs. values at 30-45 days.

and 14. The levels in DBA/2 mice on days 30–45 almost returned to control (day 0) levels, whereas in BALB/c mice they continued to be depressed. There were no significant changes in the percentage of Lyt 1+, 23+ (helper/inducer) or Lyt 1+, 23- (suppressor/cytotoxic) cells on days 7, 14, and 30–45 in the peripheral blood of either DBA/2 or BALB/c mice.

There were no significant changes in the spleen lymphocyte subsets of DBA/2 mice throughout the entire period, except for a significant decrease in Lyt 1+, 23+ (precursor) cells on day 7; however, in BALB/c mice, significant decreases were found in percentages of Lyt 1+, 23+ (precursor) cells on day 7 and Lyt 1+, 23- (helper/inducer) cells on days 7 and 14. Peripheral blood and spleen B cells in both strains showed no significant changes throughout the entire period.

In the myocardium of DBA/2 and BALB/c mice, approximately 60–80% of the cells were Thy 1.2+ (pan T) cells and 10–20% were B cells on days 7 and 14. Thereafter, levels of Thy 1.2+ (pan T) cells decreased and those of B cells increased, as measured on day 45. In both strains, Lyt 1+, 23+ (precursor) cells formed the largest T-cell subset in the myocardium on days 7 and 14; however, cells of the Lyt 1+, 23+ (helper/inducer) subset were present in the myocardium to the same extent as they were in the peripheral tissue (spleen and peripheral blood). In contrast, Lyt 1-, 23+ (suppressor/cytotoxic) cells, which form a minor subset in normal lymphoid organs, were greatly increased in the diseased myocardium on days 7 and 14. The hearts of DBA/2 and BALB/c mice were also found to show differences in their respective serial changes in lymphocyte subsets.

**Experiment II**

The precise time course of cardiac lesions in both strains has been reported elsewhere. In brief, on days 4–5 of the present experiments, necrotic foci appeared in the myocardium with small mononuclear cell infiltrations. After day 7, myocardial necrosis and inflammatory cell infiltrations became more extensive. Extensive myocardial necrosis with calcification and mononuclear cell infiltration was most severe on day 14 (Figures 3A and 3B). Dilatation of the ventricular cavity became prominent, and pleural effusion, ascites, and congestion of the lungs and liver were also noted at this time (Figure 3C). On days 30–45, myocardial fibrosis was prominent (Figure 3D), at which stage mononuclear cell infiltration was decreased (Figure 3D). The cause of death in each case was congestive heart failure.

Serial quantitative changes in the grading of positive immunoperoxidase-stained lymphocytes in the hearts of DBA/2 and BALB/c mice are listed in Table 3. The
immunohistological study of the heart confirmed the results of Experiment I that most of the stained cells in both strains were Thy 1.2 positive (pan T), and especially of the immature T cells (Lyt 1+, 23+) were occurring in either intact mice or thymectomized, irradiated mice that had been reconstituted with both thymus and bone marrow cells. Especially in antithymocyte serum-treated mice, the hearts showed only infrequent small foci of myocardial necrosis and inflammation. In encephalomyocarditis virus-infected murine models, it was also found that T cells played an important role in the severity of the induced myocarditis.17

As a result of these data, we investigated the lymphocyte subset distribution in the heart, spleen, and peripheral blood in murine viral myocarditis models by immunofluorescence techniques with laser flow cytometry (Experiment I), and we determined the histologic distribution of lymphocyte subsets in the diseased myocardium (Experiment II). The resulting study of the inflammatory sites suggested clues to the disease's pathogenesis that were not evident in the peripheral blood; thus, it may be that myocardial pan T (Thy 1.2+) and immature T cells (Lyt 1+, 23+) were involved in the development of the disease.

In Experiment I, it was found that the percentage of pan T cells (Thy 1.2+), and especially of the immature T-cell (Lyt 1+, 23+) subset, decreased in the peripheral blood but comprised the majority of infiltrating cells in the myocardium during the acute stage of the myocardial fibrosis with myocardial dysfunction in later stages.
FIGURE 3. Pathologic findings in the hearts of mice inoculated with encephalomyocarditis virus. Extensive myocardial necrosis with calcification (A, DBA/2 mouse) and marked mononuclear infiltrations (B, BALB/c mouse) are evident on day 14. Marked congestion of the lungs and liver (C) is also present at this stage. On day 45, myocardial fibrosis is prominent, and cellular infiltrations are decreased (D, DBA/2 mice). Hyper trophy of myocardial cells is also evident in D. A, B, C, and D are hematoxylin-eosin stain. Magnifications are A, x 80; B, x 370; C, x 180; D, x 180.
Table 3. Serial Changes in the Grading of Positively-Stained Lymphocytes in the Hearts of DBA/2 and BALB/c Mice After Encephalomyocarditis Virus Inoculation

<table>
<thead>
<tr>
<th></th>
<th>0 (n = 2)</th>
<th>7 (n = 6)</th>
<th>14 (n = 6)</th>
<th>30-45 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2 (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bet 1</td>
<td>—</td>
<td>—</td>
<td>10.2 ± 3.5†</td>
<td>21.5 ± 7.2</td>
</tr>
<tr>
<td>Thy 1.2 (Lyt 1 + Lyt 2)</td>
<td>—</td>
<td>46.3 ± 11.8†</td>
<td>49.2 ± 10.8†</td>
<td>21.3 ± 8.3</td>
</tr>
<tr>
<td>Lyt 1</td>
<td>—</td>
<td>42.2 ± 9.7†</td>
<td>34.3 ± 8.9*</td>
<td>19.5 ± 4.3</td>
</tr>
<tr>
<td>Lyt 2</td>
<td>—</td>
<td>34.8 ± 9.1†</td>
<td>36.2 ± 5.4†</td>
<td>18.5 ± 6.7</td>
</tr>
<tr>
<td>BALB/c (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bet 1</td>
<td>—</td>
<td>—</td>
<td>13.0 ± 6.3*</td>
<td>11.2 ± 5.7*</td>
</tr>
<tr>
<td>Thy 1.2 (Lyt 1 + Lyt 2)</td>
<td>—</td>
<td>58.5 ± 18.3†</td>
<td>62.7 ± 15.6†</td>
<td>28.5 ± 7.5</td>
</tr>
<tr>
<td>Lyt 1</td>
<td>—</td>
<td>47.2 ± 7.0‡</td>
<td>39.5 ± 9.1†</td>
<td>21.2 ± 7.5</td>
</tr>
<tr>
<td>Lyt 2</td>
<td>—</td>
<td>40.8 ± 7.4†</td>
<td>38.2 ± 8.1†</td>
<td>21.3 ± 7.1</td>
</tr>
</tbody>
</table>

Cryostat sections were stained with monoclonal antibodies and horseradish immunoperoxidase. The percentage of positively stained cells was determined blindly.

= negative; Bet 1 = B cell stain.
Values are mean ± SD; *p<0.05, †p<0.01, ‡p<0.001 vs. values at 30-45 days.

Cryostat sections were stained with monoclonal antibodies and horseradish immunoperoxidase. The percentage of positively stained cells was determined blindly.

Peripheral blood B cell counts, however, did not change significantly throughout the experiment. Thus, specific antigenic markers on lymphocytes at the site of inflammation in the acute stage of the disease differed from those on peripheral lymphocytes, and in addition, pan T cells (Thy 1.2+), most of which consisted of immature T (Lyt 1+, 23+) cells, may have been closely involved in the disease’s development. The accuracy and reproducibility of flow cytometry has already been reported, and it is unusual for a specific subset to be easily recovered by the Ficoll-Metrizoate gradient method. Thus, it is possible that the transient decrease in T-cell subsets in the peripheral blood reflected their accumulation in the diseased heart. Indeed, Reyes and Lerner have speculated that, during infections, sensitized lymphocytes migrate toward the sites of infection.

Furthermore, the two encephalomyocarditis virus—
sensitive inbred mouse strains used in this study have the same H-2 haplotype (H-2b), while other inbred strains bearing different H-2 haplotypes, for example, C57BL/6 (H-2b) and A/J (H-2a) are not susceptible to infection by this virus. Thus, susceptibility to viral infection may be primarily regulated by the histocompatibility complexes of each inbred strain. However, there were some differences between DBA/2 and BALB/c mice with behavior of T-lymphocyte subsets, especially helper/inducer (Lyt 1+, 23−) and suppressor/cytotoxic subsets (Lyt 1−, 23+) in the hearts. It has already been reported that cardiac lesions of DBA/2 mice with chronic myocarditis were very similar to those seen in some patients with dilated cardiomyopathy, but those of BALB/c mice were not; hypertrophy of myocardial cells following myocarditis were not evident in BALB/c mice but were in DBA/2 mice. Although further studies are needed, behavioral differences in the T-lymphocyte subsets in the myocardium of these mice and differences in the cardiac lesions might reflect some involvement of the genetic backgrounds affecting the response of the host to the virus, as already evidenced in lymphocytic choriomeningitis virus infection in which the host H-2 complex is only one of several genes involved.

By immunohistological analysis (Experiment II), the majority of mononuclear inflammatory cells in the diseased hearts of DBA/2 and BALB/c mice on days 7 and 14 were identified as pan T (Thy 1.2 positive) cells, most of which were Lyt 1 and Lyt 2 positive. Thereafter, on days 30–45 T cells and T-cell subsets were found to decrease in numbers, while B (Bet 1 positive) cells slightly increased; the results are consistent with those of Experiment I. The results of Experiment II may explain our previous report in which the severity of myocardial damage in T-cell-deprived BALB/c mice infected with encephalomyocarditis virus was less than that occurring in intact BALB/c mice. Some investigators conducting biopsy studies have identified cardiac immune complexes and mononuclear cell subsets in patients with myocarditis and have concluded that immunohistological studies are necessary for the accurate diagnosis of myocarditis. Experiment II also indicates that the distribution of myocardial lymphocyte subsets changed with the course of the disease.

With regard to the precise assigned functions of each murine lymphocyte subset, the Lyt 1+, 23− subset is possibly the precursor of other T-cell subsets and has been demonstrated to be an alloreactive and syngeneic cytotoxic lymphocyte subset in addition to the Lyt 1−, 23+ subset. Lyt 1+, 23− cells are not only helper/inducers in antibody and cytotoxic lymphocyte inductions but also effectors of delayed-type hypersensitiv-
FIGURE 6. Immunohistology of the heart of a BALB/c mouse on day 14. Prominent cellular infiltration is evident. Most of the stained cells are Thy 1.2, Lyt 1 and Lyt 2 positive. Arrows indicate Lyt 1 positive cells. A is hematoxylin-eosin (H.E) stain; B is Thy 1.2 positive stain; C is Lyt 1 positive stain; D is Lyt 2 positive stain. Magnifications are A, ×180; B, ×180; C, ×370, D, ×370.

Reduced suppressor T-lymphocyte activity has been noted in the peripheral blood of patients with dilated cardiomyopathy and myocarditis. However, our study suggests that investigation of the inflammatory site gives clues to the disease's pathogenesis, which cannot be found by studies of the peripheral blood. The importance of studying inflammatory site lymphocytes has also been reported for rheumatoid arthritis, kidney allograft rejection, sarcoidosis, and Sjogren's syndrome, in which lymphocytes infiltrating the target organs were shown to differ from those in the peripheral blood. What role(s) each T-lymphocyte subset plays in the pathogenesis of virus-induced myocarditis is not known at this moment. However, it is conceivable that the complex series of immune reactions, including T-cell-mediated cytotoxocities and delayed-type hypersensitivity directed against the infecting virus, which result from damage to the affected myocardium and virus elimination may aggravate the disease process. Functional studies, such as on delayed-type hypersensitivity of the infiltrating subsets, and killer-cell activities in the myocardium are now in progress using these animal models. Elucidation of the exact immune process following viral infection might shed light on the possible mechanism of transition from myocarditis to dilated cardiomyopathy.

In conclusion, our study demonstrates that the distribution of myocardial and peripheral lymphocyte subsets may change with the course of myocarditis and that specific lymphocyte subsets at the site of inflammation in the acute stage of viral myocarditis differed from peripheral lymphocytes examined at the same time. The disease state may have resulted from either selective homing of lymphocyte subsets to the inflammatory site or the clonal expansion of a subset in response to a particular antigen in the myocardium. In either event, our results emphasize that theories of viral myocarditis pathogenesis must include studies on myocardial lymphocytes. It is clear that myocardial Thy 1.2+ (pan T) and Lyt 1+,23+ (immature and T-cell subset precursors) subsets have some pathogenic role in the development of myocarditis in our experimental animals.

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Immunohistology of hearts of DBA/2 mice on days 14 and 30. Most of the stained cells are Thy 1.2 positive on day 14. Bet 1 positive (B) cells (\(\uparrow\)) are slightly increased on day 30. A is Bet 1 positive stain; B is Thy 1.2 positive stain; C is Bet 1 positive stain; D is Thy 1.2 positive stain. Magnifications are A, ×359; B, ×175; C, ×359; D, ×466.

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Immunologic identification of lymphocyte subsets in experimental murine myocarditis with encephalomyocarditis virus. Different kinetics of lymphocyte subsets between the heart and the peripheral blood, and significance of Thy 1.2+ (pan T) and Lyt 1+, 23+ (immature T) subsets in the development of myocarditis.

C Kishimoto, K Kuribayashi, K Fukuma, T Masuda, N Tomioka, W H Abelmann and C Kawai

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