Immunologic behavior of lymphocytes in experimental viral myocarditis: significance of T lymphocytes in the severity of myocarditis and silent myocarditis in BALB/c-nu/nu mice

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ABSTRACT To clarify the immune mechanism in myocarditis, we examined by immunofluorescence techniques the serial changes in percentages of T and B lymphocytes in the heart, spleen, and peripheral blood of DBA/2 mice inoculated with encephalomyocarditis (EMC) virus (experiment I). B cells were demonstrated by staining with fluorescein isothiocyanate (FITC)-labeled rabbit antimouse immunoglobulin (Ig). T cells were demonstrated with rat anti-Thy-1.2 monoclonal antibody plus FITC-labeled antimouse Ig. There was a marked decrease in T cells in peripheral blood and a moderate decrease in the cells in the spleen on day 14. There were no significant changes in B cells in peripheral blood or spleen throughout the entire period and T cells accounted for approximately 80% of the cells in the myocardium on days 7 and 14. To confirm the involvement of T cells in the development of myocarditis, we also carried out studies in which BALB/c-nu/nu mice (group 1, n = 58), BALB/c-nu/+ mice (group 2, n = 54), and BALB/c-nu/nu mice injected with 5 x 10⁷ spleen cells from BALB/c-nu/+ mice (group 3, n = 50) were inoculated with EMC virus (experiment II). Four mice from each of the three groups were killed on day 6 for virologic studies. In experiment II, there were no significant differences in the incidence of myocarditis among the three groups. Virus titrations of the heart and serum neutralizing antibody titers did not show any significant differences between the three groups on days 6 and 16. Fifty-two percent of group 2 mice (26 of 50) and 43% of those in group 3 (20 of 46) died on days 9 to 15, when congestive heart failure developed. However, only 9% of group 1 (five of 54) died during this period. Pathologic examination confirmed the presence of congestive heart failure in groups 2 and 3 but not in group 1 during this period. Cellular infiltrations and myocardial necrosis were minimal in group 1. Thus, the severity of myocarditis seems to be mediated by T cells. The so-called silent myocarditis seen clinically may be similar to myocarditis in BALB/c-nu/nu mice.


MYOCARDITIS can develop rapidly and progress to congestive heart failure. Congestive heart failure has been reported to result from many viral infections in man. Thus, a relationship between viral infection and cardiomyopathy has been suggested, but complete evidence for it is still lacking. Recently we found congestive heart failure after inducing encephalomyocarditis (EMC) virus infections in BALB/c-nu/+ mice and have also observed severe myocarditis in DBA/2 mice.

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mice. It has been reported that cardiac lesions of DBA/2 mice with chronic myocarditis are similar to those seen in some patients with dilated cardiomyopathy.

It may be that immune mechanisms play a role in the susceptibility to viral infection and in the severity of the disease. To test this hypothesis, we used an immunofluorescence method to examine the serial changes in percentages of T and B lymphocytes in the heart, spleen, and peripheral blood of DBA/2 mice inoculated with EMC virus (experiment I), and also carried out studies in which BALB/c-nu/nu mice (so-called nude), BALB/c-nu/+ mice, and BALB/c-nu/nu mice injected with spleen cells from BALB/c-nu/+ mice were inoculated with EMC virus. BALB/c-nu/nu mice are athymic and have no mature T lympho-
cytes. Inbred strains of DBA/2 and BALB/c mice have the same H-2 (H-2k). Congestive heart failure developed after EMC viral myocarditis in both strains.

Methods

**Experimental infections.** The M variant of EMC virus was used; the virus stock was prepared in cultures of human amnion (FL) cells in Eagles’ MEM. Virus suspensions were centrifuged after the cytopathic effect had developed. Virus stock had a titer of $10^6.5$ TCD$_{50}$ (50% tissue culture infective dose) determined in tissue cultures of FL cells. Virus fluid was stored at $-70^\circ$C until use.

**Experiment I.** Inbred DBA/2 mice 4 to 6 weeks of age were inoculated intraperitoneally with 0.1 ml of virus suspension containing $10^6.5$ TCD$_{50}$ per 0.1 ml. After confirming myocarditis from the gross appearance of the heart (yellowish-white patches on the surface), the heart, spleen, and peripheral blood were processed for the determination of T and B lymphocyte populations.

The heart, spleen, and the heart samples pooled from five to 10 animals, were minced gently with a sterile stainless mesh. After mincing, the cell suspension was rapidly pipetted with a sterile Pasteur pipette into 20 to 25 ml of Hanks’ balanced salt solution (HBSS) and centrifuged at 1500 rpm for 5 min. The cells and the peripheral blood collected in heparin were washed twice with HBSS. The lymphocyte fractions of these samples were obtained by Ficoll-Metz gradient centrifugation. The lymphocytes were counted in a standard hemocytometer. The cells were finally suspended at a concentration of $1 \times 10^6$ cells/ml in RPMI-1640 media with 2.5% fetal calf serum and 0.2% NaN$_3$ as the second. Percentage of B cells was determined by staining with fluorescein isothiocyanate (FITC)-labeled rabbit antimouse immunoglobulin (Ig) (Miles-Yeda). T cells were stained by use of monoclonal rat anti-Thy 1.2 (HO-13.4) as the first layer of antibodies and FITC-labeled rabbit antimouse Ig as the second.

**FITC-labeled antimouse Ig was diluted 10-fold and rat anti-Thy 1.2 monoclonal antibody 20-fold.** The (1 x $10^6$) of staining medium were put into a well of a V-bottomed 96-well culture plate and centrifuged at 1500 rpm for 3 min. After centrifugation, the cell pellet was suspended in 100 $\mu$l of staining medium or the first layer of antibodies. After incubation for 30 min at $4^\circ$C, the cells were washed three times and suspended in 100 $\mu$l of the second layer of antibodies. The cells were washed twice with staining medium after 30 min of incubation at $4^\circ$C and suspended in 50 $\mu$l of staining medium for microscopic examination. Preparations of these cells were observed under a fluorescence microscope. The percentage of positive fluorescent cells in each specimen was determined by the examination of at least 200 cells (figure 1). The percentage of T cells was obtained by subtracting the number of B cells from the cells with positive reactions to monoclonal rat anti-Thy 1.2 and FITC-labeled rabbit antimouse Ig.

**Experiment II.** The test groups consisted of 58 BALB/c-nu/nu mice (group 1), 54 BALB/c-nu/+ mice (group 2), and 50 BALB/c-nu/nu mice injected 2 weeks before the virus inoculation with $5 \times 10^7$ spleen cells of BALB/c-nu/+ mice (group 3). At 6 weeks of age, mice in these three groups were inoculated intraperitoneally with 0.1 ml of virus suspension containing $10^6.5$ TCD$_{50}$ per 0.1 ml. These mice were observed daily for 15 days after the virus inoculation. The surviving mice were killed on day 16. After gross inspection of the heart for alterations in myocardial appearance, the hearts were processed for histologic or virologic studies.

Four mice from each of the three groups were killed on day 6 for virologic study, and their hearts were processed for histologic study.

**Pathologic study.** The hearts were fixed in a 10% formalin solution, sectioned transversely at the midportion of the ventricle, embedded in paraffin, and stained with hematoxylin-eosin. The grading of the myocardial necrosis, cellular infiltration, and calcification was performed blindly on a scale of 1 + to 4 + in terms of severity. A score of 1 + represented a limited focal distribution of myocardial lesions and one of 4 + indicated the presence of multiple lesions over the entire heart, while scores of 2 + and 3 + denoted intermediate severity. The lungs, livers, kidneys, and other organs were also sectioned and stained with hematoxylin-eosin.

**Virus titration of the heart and neutralizing antibody in serum.** Hearts were ground with sand and a 1% suspension was prepared in Eagle’s MEM. The suspension was centrifuged, the supernatant was serially diluted by 10-fold increments, and 0.1 ml of each dilution was inoculated into a tube culture of FL cells containing 1.0 ml of MEM supplemented with 2% fetal calf serum. After immobilization, serum was examined by conventional techniques. Tubes were observed daily for the appearance of characteristic cytopathic effects over a period of 7 days.

**Statistical analysis.** Statistical analysis of the data was performed by an analysis of variance with multiple comparisons and the generalized Wilcoxon method. The results were expressed as mean ± SD.

**Results**

**Experiment I.** Table 1 summarizes the results of experiment I. The control (baseline) values ($n = 5$) were as follows: peripheral blood, 22.34 ± 2.49% B cells and 53.76 ± 10.67% T cells; spleen, 40.18 ± 4.19% B cells and 36.58 ± 4.68% T cells. No lymphocytes were detected in normal hearts.
TABLE 1
Serial changes in percentages of B cells and T cells and T/B cell ratio in the peripheral blood, spleen, and heart of DBA/2 mice after EMC virus inoculation (experiment 1)  

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<tr>
<td>B cell (%)</td>
<td>22.34 ± 2.49</td>
<td>24.90 ± 1.27</td>
<td>15.57 ± 1.76</td>
<td>27.63 ± 7.56</td>
<td>33.50 ± 4.88</td>
<td>33.07 ± 2.06</td>
<td>27.43 ± 2.24</td>
<td>26.37 ± 3.95</td>
<td>25.60 ± 0.85</td>
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<td>T cell (%)</td>
<td>53.76 ± 10.67</td>
<td>40.70 ± 4.24</td>
<td>40.70 ± 7.89</td>
<td>27.53 ± 8.08</td>
<td>12.03 ± 10.08</td>
<td>1.60 ± 2.77</td>
<td>36.70 ± 11.97</td>
<td>46.70 ± 8.23</td>
<td>54.10 ± 10.75</td>
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<td>T/B cell ratio</td>
<td>2.41 ± 0.64</td>
<td>1.63 ± 0.78</td>
<td>2.61 ± 0.81</td>
<td>1.00 ± 1.80</td>
<td>0.56 ± 0.96</td>
<td>0.37 ± 0.30</td>
<td>1.34 ± 0.55</td>
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<td>2.11 ± 0.49</td>
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<td>B cell (%)</td>
<td>40.18 ± 4.19</td>
<td>51.00 ± 1.41</td>
<td>47.67 ± 1.03</td>
<td>53.47 ± 6.09</td>
<td>53.13 ± 3.46</td>
<td>44.47 ± 2.04</td>
<td>53.50 ± 13.38</td>
<td>47.20 ± 4.93</td>
<td>32.95 ± 6.58</td>
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<td>T cell (%)</td>
<td>36.58 ± 4.68</td>
<td>32.10 ± 7.64</td>
<td>38.77 ± 3.61</td>
<td>22.30 ± 4.65</td>
<td>14.67 ± 13.69</td>
<td>35.70 ± 15.84</td>
<td>25.60 ± 12.12</td>
<td>26.27 ± 7.28</td>
<td>38.30 ± 4.53</td>
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<td>T/B cell ratio</td>
<td>0.91 ± 0.19</td>
<td>0.63 ± 0.17</td>
<td>0.81 ± 0.07</td>
<td>0.42 ± 0.12</td>
<td>0.28 ± 0.29</td>
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<tr>
<td>B cell (%)</td>
<td>ND</td>
<td>6.63 ± 4.48</td>
<td>6.70 ± 3.84</td>
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<td>32.87 ± 2.58</td>
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<tr>
<td>T cell (%)</td>
<td>ND</td>
<td>81.47 ± 13.40</td>
<td>77.43 ± 10.19</td>
<td>53.60 ± 4.61</td>
<td>25.33 ± 3.95</td>
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<tr>
<td>T/B cell ratio</td>
<td>12.29 ± 9.55</td>
<td>11.56 ± 6.82</td>
<td>2.27 ± 0.08</td>
<td>0.77 ± 0.15</td>
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Values are mean ± SD.

PB = peripheral blood; SP = spleen; ND = not detected.

\*p < .05; \*p < .001 vs control; \*p < .05 vs value at 7 days.

Each datum obtained from five to 10 hearts (for adequate numbers of lymphocytes).

The percentage of T cells was greatly decreased in the peripheral blood (figure 2, A) and moderately decreased in the spleen (figure 2, B) on days 10 to 15, and returned to the control levels on days 60 to 90. On day 90, T cells were 54.10% of those in the peripheral blood and 38.30% of those in the spleen. B cells in the peripheral blood and spleen did not change significantly throughout the entire period. In the myocardium, however, approximately 80% of the cells were T cells and only about 7% were B cells on days 7 and 14 (figure 2, C). Thereafter, T cells decreased and B cells increased, and on day 75 values were 25.33% T cells and 32.87% B cells. The T cell/B cell ratios are also listed in table 1.

**Experiment II**

**Mortality.** As reported previously, the mortality rate in group 2 was highest on day 4 and then decreased gradually, but increased again between days 10 and 15 (figure 3, B). Mice that died on days 9 to 15 showed pleural effusion, ascites, and congestion of the lungs and liver. The cause of death was congestive heart failure in each.

However, the mortality rate in group 1 was highest on the day 4 and decreased gradually until day 15 (figure 3, A). On days 9 to 15, only five mice died, and these mice showed no signs of congestive heart failure. In other words, no BALB/c-nu nu mice with myocarditis died of congestive heart failure during this period.

In group 3, the time course of death was similar to that in group 2 (figure 3, C), and the mice that died on days 9 to 15 in this group also showed pleural effusion, ascites, and congestion of the lungs and liver.

Fifty-two percent of group 2 (26 of 50) and 43% of group 3 (20 of 46) died on days 9 to 15, when congestive heart failure was evident. However, only 9% of group 1 (five of 54) died on days 9 to 15 (figure 4).

**Incidence and severity of myocarditis.** There were no significant differences in the incidence of myocarditis among the three groups (group 1, 82.8% [48 of 58]; group 2, 88.9% [48 of 54]; group 3, 88.0% [44 of 50]).

Table 2 shows the pathologic grades in the three groups. Cellular infiltration, myocardial necrosis, and calcification were significantly less in group 1 than in groups 2 and 3 (p < .001; group 1 vs groups 2 and 3).

**Pathologic findings.** The precise time course of cardiac lesions in group 2 has been reported elsewhere (figure 5B). In brief, on day 4 or 5, necrotic foci appeared in the myocardium with small mononuclear cell infiltrations. After day 7, myocardial necrosis and inflammatory cell infiltrations became more extensive. Dilata-
FIGURE 2. Serial changes in percentages of T and B cells in the peripheral blood (A), spleen (B) and heart (C) (experiment I). Amount of T cells was moderately decreased in the spleen and markedly decreased in the peripheral blood on day 13, whereas T cells accounted for approximately 80% of cells in the heart on days 7 and 14.

FIGURE 3. For legend see opposite page.
FIGURE 4. Survival rates in the three groups (experimental II). The survival rate in group 1 was significantly higher than that in groups 2 (p < .05) and 3 (p < .05).

FIGURE 5A. Hematoxylin and eosin stains of hearts obtained 16 days after inoculation from mice in group 1 (experiment II). a, There is no dilatation of the ventricular cavities or decrease in wall thickness (original magnification ×12). b and c, Myocardial necrosis and cellular infiltration are minimal (b, right ventricular wall, original magnification ×180; c, left ventricle, original magnification ×370).

FIGURE 3. Mortality after inoculation with EMC virus in the three groups (experimental II). The mortality rate peaked on day 4, decreased gradually, then increased again between days 10 and 15 in groups 2 and 3 (B and C). In group 1 (A), the mortality peaked on day 4, then decreased gradually until day 15. MC = myocarditis; BALB/c-nu/nu* = BALB/c-nu/nu mice injected with $5 \times 10^7$ spleen cells from BALB/c-nu/+ mice.
T lymphocytes. The course and severity of cardiac lesions in group 3 (figure 5C) were almost the same as in group 2, supporting the finding that presence of T lymphocytes is a key factor in the development of severe cardiac lesions.

**Virus titration and neutralizing antibody titers.** Virus titration of the heart and serum neutralizing antibody titers did not show any significant differences among the three groups on days 6 and 16 (table 3).

**Discussion**

In our previous studies, we found severe myocarditis in BALB/c-nu/+ mice inoculated with the M variant of EMC virus. Mice with severe myocarditis died of congestive heart failure in the acute stage of the illness. This animal preparation is considered to be an experimental model for congestive heart failure after EMC virus myocarditis. In addition, we also found severe myocarditis caused by EMC virus in an inbred strain of DBA/2 mice. Pathologic study revealed striking histologic similarities between DBA/2 mice with myocarditis in the chronic stage and some patients with dilated cardiomyopathy. This animal preparation is now considered to be an excellent one for the study of the pathogenesis of idiopathic dilated cardiomyopathy.

Resistance and susceptibility to a viral infection depend on a complex interaction of viral and host mechanisms in which the species, age, and prior immune status of the antiviral defenses mounted by the host may act either directly on viruses or indirectly on the infected cells and hence on viral replication. These defenses include local mechanisms affecting entry of

**TABLE 2**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Group 1 (n = 58^a)</th>
<th>Group 2 (n = 54^a)</th>
<th>Group 3 (n = 50^a)</th>
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<tr>
<td>+4 (n)</td>
<td>0</td>
<td>19</td>
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^aIncludes the hearts of mice killed on day 6.
^bCompared with group 1.

![FIGURE 5B](http://circ.ahajournals.org/content/1252-2017 downloading)

Hematoxylin and eosin stains of hearts obtained 16 days after inoculation from mice in group 2 (experiment II). Dilatation of both ventricular cavities and decreased wall thickness are evident (a, original magnification ×12), as are myocardial necrosis and cellular infiltration (b and c, left ventricular wall and left ventricle, respectively; respective original magnifications ×180 and ×370).
this virus, the macrophage barrier, the ability to produce antiviral antibody, and cell-mediated immunity.

Genetic factors may play a role in the susceptibility to infection and the severity of the disease. Thus, to determine the role of immunomechanisms — particularly the significance of T lymphocytes in the severity and course of the disease, we studied experimental myocarditis with use of these two animal preparations.

In experiment I, T cells decreased markedly in the peripheral blood and accounted for approximately 80% of the cells in the myocardium in the acute stage of myocarditis. The percentage of B cells did not change significantly throughout the entire period. Thus, it may be mainly T cells that are involved in the development of myocarditis in this animal preparation. This is the first serial demonstration of lymphocyte populations in experimental viral myocarditis in mice.

In experiment II, we found severe myocarditis in both BALB/c-nu/+ mice and BALB/c-nu/nu mice injected with spleen cells from BALB/c-nu/+ mice, and limited or mild myocarditis in BALB/c-nu/nu mice. In addition, we found a high death rate due to congestive heart failure on days 9 to 15 in BALB/c-nu/+ mice and BALB/c-nu/nu mice injected with spleen cells from BALB/c-nu/+ mice, but not in BALB/c-nu/nu

### TABLE 3

Virus titration and neutralizing antibody titer in the three groups (experiment II)

<table>
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<th>Group</th>
<th>Day 6 (n = 4)</th>
<th>Day 16 (n = 4)</th>
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<tr>
<td>Virus titers (log$<em>{10}$ TCD$</em>{50}$/g tissue)</td>
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<tr>
<td>Group 1</td>
<td>6.00 ± 1.08</td>
<td>ND</td>
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<td>Group 2</td>
<td>6.13 ± 1.11</td>
<td>ND</td>
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<td>Group 3</td>
<td>6.13 ± 1.31</td>
<td>ND</td>
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<td>Antibody titers (log 2)</td>
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<td>Group 1</td>
<td>5.88 ± 0.85</td>
<td>10.25 ± 1.66</td>
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<td>Group 2</td>
<td>6.38 ± 1.55</td>
<td>10.88 ± 1.38</td>
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<td>Group 3</td>
<td>6.13 ± 0.75</td>
<td>10.25 ± 1.71</td>
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ND = not detected (all values are less than 2 log$_{10}$ TCD$_{50}$/g tissue).
mice. Death on days 4 to 8 was more likely to be the result of conduction disturbances, particularly complete atrioventricular block, as we have already reported. Cellular infiltration, myocardial necrosis, and calcification were minimal in BALB/c-nu/nu mice with myocarditis. It is clear that the development of cardiac dilatation depends on the severity of the myocarditis. Myocardial virus titration and serum antibody titers in the three groups showed that viral clearance from the myocardium is controlled by B lymphocytes, which are involved mainly in humoral immunity since EMC virus is a picornavirus biologically similar to coxsackievirus. This study supports the view that development of effective anti-EMC viral resistance occurs independently of T cell function. Experiments I and II show that the severity and development of myocarditis may depend on T lymphocytes, which control cell-mediated immunity.

Recently, T cell–mediated immunity had been reported in the pathogenesis of coxsackievirus B myocarditis in mice. The severity of myocarditis is reduced in animals depleted of T cells, and studies in vitro have shown that infection stimulates the production of cytotoxic T cells. Huber and his colleagues reported that cytotoxic T lymphocytes were involved in the pathogenesis of the myocyte damage that occurs in coxsackievirus myocarditis in mice. To understand the significance of cytotoxic T cells in vivo, however, we must consider which other cells are involved in their activation.

Reduced suppressor activity has been reported in patients with dilated cardiomyopathy and myocarditis. Although further studies are needed, this animal preparation of BALB/c-nu/nu (athymic) mice with EMC virus infection may represent a condition comparable to that of patients with so-called silent myocarditis. In other words, the spectrum from silent to severe myocarditis depends on the immune status of individual patients, even when they have the same susceptibility to viral infection. It may be that the incidence of silent myocarditis is higher than expected.

In conclusion, it seems that the severity and development of myocarditis was mediated by T cells in our experimental preparations as well as in those used in previous experiments.

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