Immunological Mechanisms in Experimental Coxsackievirus B3 Myocarditis in Mice

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To address unresolved questions, experimental models of viral myocarditis may be of great value. In this study, immunological mechanisms of myocardial damage in coxsackievirus B3 myocarditis in mice were investigated. The results showed that susceptibility to viral infection is primarily determined by the genetic background of the host, that the severity of myocarditis depends not upon B cells but upon T cells, and that antigen-specific T cells play a pivotal role in the pathogenesis of acute coxsackievirus B3 myocarditis.

The hypothesis that currently best fits what is known about the natural history of viral myocarditis suggests that the initial cardiac damage and manifestations are due to viral replication in heart muscle, and that the extent of damage is determined not only by the cardiotropism and virulence of the virus, but also by the genetic and immunologic characteristics of the host.

The phase of viral replication in the heart, however, is short (1 to 2 weeks at most), and the subacute and chronic active phases of myocarditis are thought to be a function of autoimmune or immune processes. To address some of these unresolved questions, an experimental model of viral myocarditis, namely, coxsackievirus B3 myocarditis, may be of great value. In this study, immunological mechanisms of myocardial damage in murine coxsackievirus B3 myocarditis were investigated.

Key words:
Murine myocarditis
Coxsackievirus B3
Antigen-Specific T cells

METHODS

Viruses: The Nancy strain of coxsackievirus B3 (CB3), and the myocardiotropic variant of encephalomyocarditis virus (EMC) were used; the viral stock was prepared in cultures of kidney cells the African green monkey in Eagle’s minimum essential medium. A cell-conditioned medium containing suspensions of the virus was centrifuged after the cytopathic effect had developed. Viral stocks were stored at $-70^\circ C$ until used. The precise method of experimental infection has been described previously.

Experiment 1

Two-week-old male C57BL/6 (n=9), DBA/2 (n=9), BALB/c (n=9), A/J (n=6), and C57BL/6 (n=6) mice were inoculated intraperitoneally with $3 \times 10^5$ plaque forming units (pfu) of CB3. The mice were observed daily for 12 days after inoculation with the virus and the surviving mice were killed on
TABLE I INCIDENCE, COURSE, AND PATHOLOGIC CHARACTERISTICS OF MYOCARDITIS IN INBRED STAINS OF 2-WEEK-OLD MICE INOCULATED WITH $3 \times 10^5$ PLAQUE FORMING UNITS OF COXSACKIEVIRUS B3

<table>
<thead>
<tr>
<th>Mice</th>
<th>Histo-compatibility</th>
<th>Survival rate* (%)</th>
<th>Incidence of myocarditis (%)</th>
<th>Myocardial pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>H-2a</td>
<td>4/6 (66.7)</td>
<td>3/6 (50)</td>
<td>moderate  moderate  moderate</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>H-2b</td>
<td>2/6 (33.3)</td>
<td>1/6 (16.7)</td>
<td>mild     mild     mild</td>
</tr>
<tr>
<td>BALB/c</td>
<td>H-2d</td>
<td>9/9 (100)</td>
<td>9/9 (100)</td>
<td>mild     moderate mild</td>
</tr>
<tr>
<td>DBA/2</td>
<td>H-2d</td>
<td>8/9 (88.9)</td>
<td>9/9 (100)</td>
<td>moderate moderate moderate</td>
</tr>
<tr>
<td>C3H/He</td>
<td>H-2e</td>
<td>8/9 (100)</td>
<td>9/9 (100)</td>
<td>severe   severe   severe</td>
</tr>
</tbody>
</table>

*Observed on day 12.

TABLE II SERIAL CHANGE IN THE FRACTION (%) OF POSITIVELY STAINED LYMPHOCYTES IN THE HEART OF C3H/He MICE AFTER COXSACKIEVIRUS B3 INOCULATION

<table>
<thead>
<tr>
<th></th>
<th>Day 0 (n=1)</th>
<th>Day 7 (n=4)</th>
<th>Day 14 (n=4)</th>
<th>Day 30 (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bet 1</td>
<td>(%)</td>
<td>3.5±0.6</td>
<td>2.1±1.0</td>
<td>8.3±2.1**</td>
</tr>
<tr>
<td>Thy 1.2 (Ly 1+Ly 2)</td>
<td>(%)</td>
<td>38.9±14.6</td>
<td>44.4±12.1</td>
<td>23.1±10.3</td>
</tr>
<tr>
<td>Ly 1</td>
<td>(%)</td>
<td>13.0±10.0</td>
<td>41.9±8.7</td>
<td>12.7±9.1*</td>
</tr>
<tr>
<td>Ly 2</td>
<td>(%)</td>
<td>19.9±7.5</td>
<td>32.5±17.6</td>
<td>16.4±5.7</td>
</tr>
<tr>
<td>L3T4</td>
<td>(%)</td>
<td>9.2±5.4</td>
<td>13.7±3.1</td>
<td>11.1±4.7</td>
</tr>
</tbody>
</table>

(mean±S.D.)

Cryostat sections were stained with monoclonal antibodies and horseradish immunoperoxidase technique. The percent of positively stained cells was determined in a blind manner. For Thy 1.2 of Ly 1+Ly 2 population, higher value was cited.

— = negative, Bet 1 = B cell, Thy 1.2 = pan T cell, Ly 1 = precursor of the other T cell subsets, Ly 2 = suppressor/cytotoxic T cell, L3T4 = helper T cell.

*p<0.05, **p<0.001 vs values at 7 day.

day 12. The hearts were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylin-eosin. Myocardial necrosis, cellular infiltration and calcification were observed.

Experiment II

Three-week-old male C3H/He mice were inoculated intraperitoneally with CB3 containing $3 \times 10^3$ pfu/0.1 ml. After confirmation of the presence of myocarditis, the heart were processed on days 7 (n=4), 14 (n=4) and 30 (n=4) for immunohistologic study. Six micron sections were cut from the frozen blocks on a cryostat at -20°C, placed on glass slide, air-dried for 1 h, and fixed in 95% cold methanol. Cell surface markers were demonstrated in situ by indirect immunoperoxidase staining. For quantitation of lymphocyte subsets, the number of lymphocytes in each section that were stained by each monoclonal antibody was recorded along with the total number of nucleated cells, and the percentage of stained lymphocytes was then calculated.

Experiment III

Four-week-old male BALB/c-nu/nu (athymic) and BALB/c-nu/+ ( euthymic) mice were inoculated intraperitoneally with $10^8$ pfu of CB3. Ten days post-infection, the mice were divided into 6 groups, and treated as follows: Group A: nu/nu. Group B: nu/+ , Group C: nu/nu injected with $5 \times 10^7$ spleen cells of nu/+ infected 2 weeks before with CB3. Group D: nu/nu injected with

TABLE III RESULTS OF MYOCARDIAL HISTOLOGY AND IMMUNE-STAINING

<table>
<thead>
<tr>
<th>Groups</th>
<th>Severity of myocardial lesions (1+ to 4+)</th>
<th>Grade of immunoperoxidase staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infiltration</td>
<td>Necrosis</td>
</tr>
<tr>
<td>A</td>
<td>0.79 ± 0.57</td>
<td>0.64 ± 0.48</td>
</tr>
<tr>
<td>B</td>
<td>1.92 ± 0.60*</td>
<td>1.42 ± 0.47*</td>
</tr>
<tr>
<td>C</td>
<td>1.44 ± 0.73*</td>
<td>1.28 ± 0.57*</td>
</tr>
<tr>
<td>D</td>
<td>0.78 ± 0.36</td>
<td>0.67 ± 0.50</td>
</tr>
<tr>
<td>E</td>
<td>0.94 ± 0.53</td>
<td>0.72 ± 0.51</td>
</tr>
<tr>
<td>F</td>
<td>1.06 ± 0.58</td>
<td>0.56 ± 0.39</td>
</tr>
</tbody>
</table>

*p<0.05 vs Group A.

For characterization of groups, see text in detail.

Severity of myocardial histopathology was evaluated on days 10–18. Immunoperoxidase stainings were done in mice sacrificed on day 18; reactivity: (−) nonreactive; (1+) ≤ 5%; (2+) ≤ 10%; (3+) ≤ 25%; (4+) ≤ 50% of infiltrating small nucleated cells.

\[10^7\] splenocytes of nu/+ infected 2 weeks before with EMC. Group E: nu/nu injected with \[5 \times 10^7\] splenocytes of nu/nu infected 2 weeks before with CB3, and Group F: nu/nu injected with \[5 \times 10^7\] anti-Thy 1.2 antibody plus complement treated splenocytes of nu/+ infected 2 weeks before with CB3. Mice were observed daily until the 18th day post-infection, and when found dead, complete necropsies were performed. Surviving mice were sacrificed by bleeding from the retro-orbital plexus on day 18. After gross inspection, the hearts were processed for histologic and immunopathologic studies. Hematoxylin-eosin and indirect immunoperoxidase staining procedures were performed similarly, as described before.

Statistical analysis: Statistical analysis of the data was performed by analysis of variance with multiple comparisons. A probability value of less than 0.05 was considered indicative of a statistically significant difference.

RESULTS

Experiment I (Table I)

The survival rate, incidence of acute myocarditis, and pathologic characteristics (including necrosis, cellular infiltration and myocardial calcification) after infection with CB3 are listed in Table I. Considerable variation was seen in mortality as well as in the incidence and severity of myocarditis. Necrosis, infiltration, and calcification ranged from mild to severe in the different strains of mice studied.

Experiment II (Table II)

The fractions of myocardial lymphocytes of each subset stained by immunoperoxidase at each point in time are listed in Table II. Most of the stained cells on days 7 and 14 were Thy 1.2, Ly 1, and Ly 2; L3T4 positive cells were relatively few in this period. On day 30, quantities of Thy 1.2, Ly 1, and Ly 2 positive cells were decreased. Notably, Ly 1 positive cells were more prominent on days 7 and 14 than Ly 2 positive cells, but the latter become more prominent than the former on day 30. Bet 1 positive cells were quite infrequent by found on days 7 and 14, but were more common on day 30. There were no distinct differences in the anatomic distributions of each lymphocyte subset in the diseased myocardium.

Experiment III (Table III)

The incidence of myocarditis was 100% in each infected group. No differences in the severity of myocardial lesions were noted in the 6 infected groups until day 10. However, after the reconstitution treatment, cellular infiltration and myocardial necrosis were significantly more severe in Group B and C than in Group A (p < 0.05). In Group B (nu/+), most of the stained cells were Thy 1.2, Ly 1 or Ly 2. Bet 1 positive cells were quite rare. No positive T cells or T cell subsets were noted in the diseased myocardium.
in Groups A (nu/nu) and D. The presence of Thy 1.2 and Ly 1 positive cells was documented in the myocardium of Group C, the animals of which were originally T cell-depleted, suggesting that the presence of virus-specific T cells is a key factor in the development of severe cardiac lesions.

DISCUSSION

It has been proposed that experimental infection of mice with CB3 results in a biphasic disease process, and that the pathogenetic mechanism responsible is different in the two phases\textsuperscript{3,4}. The myocardial inflammation and necrosis observed during the acute phase has been attributed directly to viral replication in the myocardium, whereas the subsequent subacute and chronic inflammatory reaction, which results in progressive myocyte damage, hypertrophy and eventually, in ventricular dilatation and failure, has been attributed to cell-mediated immune responses to a neoantigen developed during the preceding acute phase.

The data presented here indicate that the susceptibility of a virus infection is determined primarily by the genetic background of the host, that T cells but not B cells play a role in the development of myocarditis, and that antigen specific T cells play a pivotal role in the pathogenesis of CB3 myocarditis, namely, T cells aggravate the disease process, provided that they have been sensitized by the same virus.

In Experiment I, striking differences were observed in the incidence, severity, and mortality of acute myocarditis among different inbred strains of mice in the face of challenge with the same cardiotropic virus. The virus-host interaction may determine not only disease susceptibility, but also myocardial responses and the characteristics of myocardial damage. The results are in agreement with previous studies\textsuperscript{5,6}.

In Experiment II, most of the mononuclear inflammatory cells in the diseased heart of C\textsubscript{3}H/He mice on days 7 and 14 were identified as Thy 1.2 positive (pan T cell); most of them were Ly 1 and Ly 2 positive. Thereafter, T cells and their subsets decreased in number, and B cells (Bet 1 positive) increased slightly in number on day 30. These results may explain the previous finding that the severity of myocardial damage in T cell-deprived BALB/c mice infected with CB3 was less than that occurring in intact BALB/c mice\textsuperscript{7}

The results of Experiment II show that not T cells sensitized by a different virus, but same-virus sensitized T cells, mainly T 1.2 positive, Ly 1 and Ly 2 positive subsets (immature T), appear to play a pivotal role in the pathogenesis of CB3 induced myocarditis. Thus, nu/nu mice reconstituted with specific-virus sensitized T cells showed severe myocarditis. In contrast, in nu/nu mice reconstituted with T cells sensitized by a different virus, myocardial damage remained mild. Indeed, evidence is accumulating that cytotoxic T lymphocytes capable of lysing virus-infected myofibers and fibroblasts are produced in CB3 infected mice\textsuperscript{8} From our data, these cells appear to recognize viral specificities, and may thus be considered to have receptors for virus modified H-2 antigens.

The study also strongly supports the concept that the myocardial cellular infiltrates adjacent to myopathic myocardium in the post-viremic, subacute phase of this experimental model of CB3 myocarditis do not constitute an epiphphenomenon, but play a pathogenetic role in the ongoing myocardial damage. This response, however, may require sensitization by a specific virus.

In conclusion, immature but antigen-specific T cells play a role in the pathogenesis of myocarditis. The results of the present study indicate that both undifferentiated (immature) and antigen-specific T cells are responsible for progressive tissue damage in the infected heart.

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