Expression of Perforin in Infiltrating Cells in Murine Hearts With Acute Myocarditis Caused by Coxsackievirus B3

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Background. Cell-mediated autoimmunity has been strongly implicated in the pathogenesis of viral myocarditis.

Methods and Results. Using a murine model of acute myocarditis caused by coxsackievirus B3, we analyzed the phenotypes and morphology of the infiltrating cells in the hearts by immunofluorescence and electron microscopy. We also examined the expression of a cytolytic factor, perforin, in the infiltrating cells by immunoperoxidase and in situ hybridization. We found that the dominant population of the infiltrating cells were asialo GM1 positive, were negative for T-cell markers, and had electron-dense cytoplasmic granules, which is consistent with a morphology of large granular lymphocytes. Perforin was found in the cytoplasmic granules of the infiltrating cells expressing perforin messenger RNA. These findings provide for the first time the direct evidence that the first wave of cell infiltration in hearts mainly consists of killer cells and strongly suggests that perforin plays, in part, an important role in myocardial cell damage involved in acute viral myocarditis. T-helper cells and cytotoxic T lymphocytes made up the second wave of cell infiltration.

Conclusions. As we previously reported, the expression of major histocompatibility complex class I antigen on cardiac myocytes induced by the infiltrating cells, such as killer cells, may facilitate the interaction between cardiac myocytes and cytotoxic T lymphocytes, and may lead to further myocardial cell damage in a later phase. (Circulation 1991;84:788–795)

In humans, viral myocarditis not only can cause congestive heart failure as an acute inflammatory disorder, but has also been strongly implicated in the pathogenesis of idiopathic dilated cardiomyopathy. The pathogenesis of the myocardial cell damage involved is of great clinical significance and remains to be clarified. Many studies have been performed in this field using murine models of myocarditis caused by coxsackievirus B3 (CVB3) and have shown that T lymphocytes may play an important role. In particular, cytotoxic T lymphocytes have been proposed as the major effector cells involved in the immunopathology. Other studies have demonstrated that natural killer cells are activated in the early stage of this disease and provide some protective effects by limiting virus replication. Cytotoxic T lymphocytes and natural killer cells are thought to kill virus-infected cells or tumor cells with the effector molecules contained in their cytoplasmic granules, one of which is pore-forming protein or perforin.

In this study, first, we analyzed the phenotypes and morphology of the infiltrating cells in the hearts of C3H/He mice on day 7 of CVB3 infection by immunofluorescence and electron microscopy. Second, to determine whether cell-mediated cytoxicity really occurs in CVB3-induced acute myocarditis, we examined the expression of perforin in the infiltrating cells with immunoperoxidase and in situ hybridization techniques. Here, we show that perforin was detected in natural killer–like large granular lymphocytes, which represent the main infiltrating cell type in the murine hearts with acute myocarditis caused by CVB3.
Methods

Virus and Animals

CVB3 (Nancy strain), a gift from Dr. Y. Kitaura (Osaka Medical College, Osaka), was grown in FL cells (human amnion), which were supplied by the Japanese Cancer Research Bank—Cell Bank (National Institute of Hygienic Sciences, Tokyo). The virus preparation had a titer of $1 \times 10^8$ plaque-forming units (PFU)/ml and was stored at $-70^\circ$C. Seven-week-old C57/He male mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were inoculated intraperitoneally with $5 \times 10^8$ PFU CVB3 in 0.2 ml phosphate-buffered saline (PBS).

Antibodies

Polyclonal rabbit anti-asialo GM1 antibody was purchased from Wako Pure Chemical Industries, Ltd. (Osaka), and was also supplied by Yamasa Shoyu Co., Ltd. (Chiba, Japan). Anti-Thy 1 monoclonal antibody (hybridoma 4A3) was a gift from Dr. T. Nishimura (Tokai University, Kanagawa, Japan). Anti-L3T4 monoclonal antibody (RM4-5) and anti-Lyt 2 monoclonal antibody (SH10-1) were gifts from Dr. K. Takahashi (Juntendo University, Tokyo). Anti-CD3 monoclonal antibody (2C11) was a gift from Dr. J.A. Bluestone (National Cancer Institute, Bethesda, Md.). Anti-F4/80 (macrophage surface antigen) monoclonal antibody was purchased from Serotec Ltd (Oxford, England). The procedures for preparing rat anti-mouse perforin monoclonal antibody were described in detail elsewhere. Briefly, rats were immunized with a polypeptide fragment of recombinant mouse perforin, which was prepared by transfecting a mouse perforin complementary DNA fragment into Escherichia coli.

Immunofluorescence

Mice were killed on day 7 after virus inoculation. Cryostat sections (6-μm thick) of heart ventricles were prepared, air dried, and fixed in acetone for 5 minutes at 4°C. After washing three times in PBS for 15 minutes, the serial sections were blocked with 2% goat or rabbit serum in PBS for 30 minutes at 37°C, then incubated with rabbit anti-asialo GM1 antibody; rat anti-Thy 1, anti-L3T4, anti-Lyt 2, and anti-F4/80 monoclonal antibodies; and hamster anti-mouse CD3 monoclonal antibody for 1 hour at 37°C. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin G antibody (United Biomedical, Inc., Lake Success, N.Y.) for anti-asialo GM1 antibody with biotinylated rabbit anti-rat immunoglobulin G antibody (Vector Laboratories, Inc., Burlingame, Calif.) for anti-Thy 1, anti-L3T4, anti-Lyt 2, and anti-F4/80 antibodies, and with biotinylated goat anti-hamster immunoglobulin G antibody (Caltag Laboratories, Inc., South San Francisco, Calif.) for anti-mouse CD3 antibody for 1 hour at 37°C. After washing in PBS, the sections were incubated with fluorescein isothiocyanate (FITC)–conjugated avidin D (Vector Laboratories) for 30 minutes at 37°C. After washing in PBS, coverslips were mounted with glycerin. The sections were examined and photographed under a fluorescence microscope (Microphot-FX, Nikon, Tokyo). Using spleen tissue samples as positive controls, we confirmed that the potential of the antibody to recognize the antigens was not altered by acetone fixation (data not shown).

Electron Microscopy

Ventricles of mice killed on day 7 of infection were fixed in 2% glutaraldehyde in PBS at 4°C, postfixed in 2% osmium tetroxide in PBS at 4°C, dehydrated in ethanol, and embedded in Epok 812 resin (Ouken Shoji, Co., Ltd., Tokyo). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with an electron microscope (JEM-1200EX, JOEL Ltd., Tokyo).

Immunoperoxidase

Cryostat sections of ventricles of mice killed on day 7 of infection were fixed in acetone for 3 minutes at 4°C and were washed in PBS and then in 4% paraformaldehyde in PBS for 1 minute at room temperature. After washing in PBS, the sections were incubated in 0.3% H2O2 in methanol for 20 minutes, washed again in PBS, and treated with 0.5% periodic acid for 10 minutes at room temperature. After washing in PBS, the sections were blocked with 2% rabbit serum in PBS for 30 minutes at 37°C, then incubated with rat anti-mouse perforin monoclonal antibody for 1 hour at 37°C, washed in PBS, and incubated with biotinylated anti-rat immunoglobulin G antibody (Cappel Laboratories, Malvern, Pa.), which was preabsorbed with mouse sera, for 1 hour at 37°C. After washing in PBS, the sections were incubated with avidin-biotinylated peroxidase complex (ABC-immunoperoxidase kit, Vector Laboratories) according to the manufacturer's instructions for 30 minutes at 37°C, washed in PBS, and reacted with diaminobenzidine tetrahydrochloride (0.2 mg/ml). After washing in PBS, the sections were counterstained with hematoxylin and then dehydrated in ethanol, and the coverslips were mounted in xylene with resin.

Preparation of Labeled RNA Probes

A cDNA fragment of about 0.94 kilobasepairs of the 5' end of the mouse perforin gene was subcloned into the EcoRI site of pBluescript SK(+) vector (Stratagene, La Jolla, Calif.). After linearization of the plasmid with appropriate restriction enzymes, sulfur-35–labeled antisense and sense RNA probes (2.9×10^7 and 2.7×10^6 cpm/μg cDNA) were synthesized by T3 and T7 RNA polymerase, respectively, with 35S-UTP (Amersham Japan Ltd., Tokyo), an unlabeled mixture of (ATP, GTP, and CTP), dithiothreitol, and human placental ribonuclease inhibitor.

In Situ Hybridization

Cryostat sections (6 μm thick) of ventricles of mice killed on day 7 of infection were prepared on slides, which were pretreated in 3×SSC (1×SSC; 0.15 M sodium chloride and 0.015 M sodium citrate), Den-
FIGURE 1. Phenotypic analysis of infiltrating cells. Serial sections of hearts of mice killed on day 7 of coxsackievirus B3 infection were stained with anti-asialo GM1 (panel A), anti-Thy 1 (panel B), anti-CD3 (panel C), anti-L3T4 (panel D), anti-Lyt 2 (panel E), and anti-F4/80 (panel F) antibody. Most infiltrating cells were positive for asialo GM1 (panel A) and negative for Thy 1, CD3, L3T4, Lyt 2, and F4/80 antigens (panels B to F). Original magnification, ×400.

hart's solution containing 0.02% Ficoll-400, 0.02% polyvinylpyrrolidone-360, and 0.02% bovine serum albumin. The sections were then air dried and fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. After washing three times in PBS for 15 minutes, the sections were dehydrated in ethanol and stored at −20°C until use. The sections were washed three times in 2×SSC for 15 minutes, incubated in 0.1 M glycine and 0.1 M Tris-HCl, pH 7.0, for 30 minutes at room temperature, and washed in 2×SSC for 15 minutes. Prehybridization was carried out overnight at 50°C in a solution containing 50% deionized formamide, 2×SSC, 0.05 M 2-mercaptoethanol, 1 mg/ml transfer RNA, 2 mg/ml methylated bovine serum albumin, and 1 mg/ml denatured salmon sperm DNA. The sections were hybridized with 35S-labeled antisense or sense mouse perforin RNA probe in the same solution overnight at 50°C, washed six times in 50% formamide and 2×SSC for 3 hours at 50°C, and washed twice in 2×SSC for 1 hour at room temperature. Then, they were dipped into NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, N.Y.), which was diluted 1:2 with 6 M ammonium acetate. After exposure for 4 days at 4°C,
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FIGURE 2. Electron photomicrograph of the infiltrating cells in hearts of mice killed on day 7 of coxsackievirus B3 infection. Most infiltrating cells had electron-dense large cytoplasmic granules (arrows), consistent with the morphology of large granular lymphocytes. N, nucleus. Bar, 5 μm.

the sections were developed and fixed with GBK developer and fixer (Kodak), then counterstained with hematoxylin and dehydrated in ethanol, and the coverslips were mounted in xylene with resin.

Results

Phenotypic and Morphological Analyses of the Infiltrating Cells

As shown in Figure 1, most of the infiltrating cells in the hearts of mice on day 7 of CVB3 infection were asialo GM1 positive (Figure 1A), negative for Thy 1 and other T-cell markers (CD3, L3T4, and Lyt 2) (Figure 1, B–E), and also negative for F4/80 as a macrophage marker (Figure 1F). This suggests that most of the infiltrating cells were natural killer–like cells from the phenotypes of surface antigens because asialo GM1 is mainly expressed on natural killer cells and may also be present on activated T cells and macrophages.10–12 The relative distribution of asialo GM1, L3T4, and Lyt 2 among the infiltrating cells is shown in Table 1. The predominant markers found on the infiltrating cells were asialo GM1, which represented nearly 90% of the infiltrating cells in the earlier stage. Asialo GM1 positive cells decreased sharply during the course of acute myocarditis. Instead, L3T4 positive cells and Lyt 2 positive cells increased in the later stage and represented 10–20% of the infiltrating cells.

Morphologically, natural killer cells are known to be mononuclear cells with large cytoplasmic granules, consistent with large granular lymphocytes. We examined the ultrastructure of the infiltrating cells by electron microscopy (Figure 2) and found that most of the infiltrating cells had electron-dense large granules in the cytoplasm (Figure 2, arrows) with a nucleus of high cytoplasm-to-nucleus ratios. We also found that some of the infiltrating cells were in close contact with myofibers (data not shown).

TABLE 1. Relative Distribution of Phenotypic Markers Among Infiltrating Cells in Murine Hearts With Acute Viral Myocarditis

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Asialo GM1</th>
<th>L3T4</th>
<th>Lyt 2</th>
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<tbody>
<tr>
<td>5</td>
<td>90</td>
<td>3</td>
<td>2</td>
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<tr>
<td>7</td>
<td>85</td>
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<td>15</td>
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<td>18</td>
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Four hundred cells were counted for each marker.
Thus, the phenotypic and morphological analyses revealed that most of the infiltrating cells were natural killer–like large granular lymphocytes in the early stage of acute myocarditis.

Expression of Perforin in the Infiltrating Cells

Figure 3 shows immunoperoxidase staining of the hearts on day 7 of CVB3 infection with anti-mouse perforin monoclonal antibody. Some of the infiltrating cells were clearly stained in their peripheral cytoplasm, indicating the expression of perforin in their cytoplasmic granules (Figure 3, arrows). There was apparent heterogeneity in the expression of perforin among different infiltrating cells. The proportion of perforin-positive cells as a fraction of total cells was 13% to 15% on day 5 of CVB3 infection, when massive cell infiltration began to appear. This was followed by tendency of gradual decrease.

Detection of Perforin mRNA in the Infiltrating Cells by In Situ Hybridization

To confirm the expression of perforin at the transcriptional level, we analyzed the expression of perforin messenger RNA by an in situ hybridization technique using 35S-labeled antisense and sense RNA probes. Figure 4 shows the results of in situ hybridization of serial sections of the hearts on day 7 of CVB3 infection with antisense (Figure 4A) or sense (Figure 4B) RNA probe. Strong signals of perforin gene transcripts were found on many infiltrating cells (Figure 4A), and the number of the cells expressing the perforin gene was much more than that of perforin antigen (data not shown). Hybridization with a sense RNA probe as a negative control revealed no significant level of signals (Figure 4B), showing that the nonspecific background was low.

Discussion

In this study, we demonstrated that the dominant population of the infiltrating cells in murine hearts with acute myocarditis caused by CVB3 were natural killer–like large granular lymphocytes and that they expressed a cytolysic factor, perforin.

Up to now, many studies have been performed in this field and have shown that T cells may play an important role in the pathogenesis of myocardial cell damage. However, there has been no evidence showing that most of the infiltrating cells had T-cell specific surface antigens such as CD3 or T-cell receptors in the early stage of this disease. In this study, we showed that the dominant population of the infiltrating cells was asialo GM1 positive and negative for T-cell markers. An antibody for NK 1.1 antigen, which is a more specific marker than asialo GM1 for natural killer cells, had been developed.13–16 However, because the potential of this antibody to detect
the antigen is limited to certain strains of mice (not including C3H/He mice), we could not use this antibody in this study. To determine whether NK 1.1 antigen is expressed on the infiltrating cells in acute myocarditis, further studies using other strains of mice are required. Electron microscopic analysis also demonstrated that most of the infiltrating cells were natural killer–like cells. Our data showed that natural killer cells mainly made up the first phase of the infiltrating cells and that T cells, which recognize antigen-presenting cells in a manner restricted by syngeneic major histocompatibility complex antigens, made up the second phase. We previously reported that major histocompatibility complex antigens, especially class I antigen, were strongly expressed on cardiac myocytes in this model of acute viral myocarditis, and this suggested that enhanced expression of major histocompatibility complex class I antigen may facilitate the interaction between the cardiac myocytes and cytotoxic T lymphocytes.17 Recently, with an in situ hybridization technique, we found that most of the infiltrating cells in the hearts 7 days after virus infection were synthesizing cytokines such as interferon-γ and tumor necrosis factor-α (unpublished data). This supported that natural killer cells, which made up the first wave of cell infiltration, induced the expression of major histocompatibility complex antigens on adjacent cardiac myocytes by the release of cytokines and may prepare the cardiac myocytes for the interaction with T cells. Persistent expression of major histocompatibility complex antigens may lead to long-term myocardial cell damage by cytotoxic T lymphocytes in the late phase. Although T-cell responses were rather dominant, similar kinet-ics of the immune cell responses were also shown in lymphocytic choriomeningitis virus infection.18 It seems likely that the strength of natural killer cell and T-cell responses depends on the type and dose of viruses and the strain of mice. Yamaki and his colleagues19,20 demonstrated the sequential infiltration of various phenotypes of cells into tumor tissues in rats inoculated with T-9 gliosarcoma. They showed that, first, neutrophils infiltrated into tumor tissues and produced a T-helper cell-specific chemotactic factor (lymphocyte migration factor-a); then, second, T-helper cells infiltrated and produced a cytotoxic T lymphocyte-specific lymphocyte migration factor (lymphocyte migration factor-b), and, third, cytotoxic T lymphocytes infiltrated into tumor tissues. For another recent example of autoimmune diseases, Hutchings et al.21 showed that blockade of macrophage complement receptor type-3 in vivo in nonobese diabetic mice prevented intraislet infiltration by both macrophages and T cells and inhibited

**FIGURE 4.** Detection of perforin messenger RNA in infiltrating cells by in situ hybridization. Serial sections of hearts of mice killed on day 7 of coxsackievirus B3 infection were hybridized with 35S-labeled antisense (panel A) or sense (panel B) mouse perforin RNA probe. Many infiltrating cells expressed perforin mRNA (panel A). Hybridization with a sense RNA probe as a negative control showed no significant level of signals (panel B). Original magnification, ×800.
development of insulin-dependent diabetes mellitus. They suggested that the early phase of macrophage migration into the islets promoted later intrasilet T-cell migration by local production of cytokines or by unknown mechanisms. Although these studies each approached a different pathogenesis, their data support the kinetics of the immune cell infiltration into the site of inflammation as revealed in the present study.

Natural killer cells and cytotoxic T lymphocytes are known to contain perforin in their cytoplasmic granules in vitro. However, until recently, there has been no evidence showing the role of perforin in cell-mediated cytotoxicity primed in vivo. Young et al. demonstrated the expression of perforin mainly in CD8 positive cytotoxic T lymphocytes and in a small population of natural killer–like cells during lymphocytic choriomeningitis virus infection. Later, this was confirmed at the transcriptional level by in situ hybridization. The investigators reported that about 15% of total CD8 positive cells expressed perforin antigen, and this is consistent with the result obtained in asialo GM1 positive cells in the present study. We also found that the number of the cells expressing the perforin gene was much more than that of the perforin antigen (data not shown). This seems to be due to the cells that have just released perforin and have not yet contained detectable levels of perforin antigen for the immunoperoxidase technique. We also could not exclude the possibility that the anti-perforin monoclonal antibodies could not reach all of the antigens or that there was a low level of expression of the perforin antigen below the limit of sensitivity of the immunoperoxidase technique. Our study demonstrates that perforin was present in asialo GM1 positive large granular lymphocytes that appeared in the hearts early on CVB3 infection. We also found that Lyt 2 positive cells began to appear in some portion of the infiltrating cells in the second phase, with its peak later than day 11 of CVB3 infection. Whether or not perforin is expressed in these cells is currently under investigation.

This study provides for the first time the direct evidence that the first wave of cell infiltration in the hearts mainly consists of killer cells and strongly suggests that perforin plays, in part, an important role in the development of myocardial cell damage involved in acute viral myocarditis.

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