Expression of Major Histocompatibility Complex Class I Antigen in Murine Ventricular Myocytes Infected With Coxsackievirus B3

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Evidence has accumulated that T cell–mediated autoimmunity plays an important role in the pathogenesis of viral myocarditis. T lymphocytes are known to recognize antigen-presenting cells, such as virus-infected cells, being restricted by syngeneic major histocompatibility complex (MHC) antigens. To clarify in more detail the immunological mechanisms involved, we induced acute viral myocarditis in C3H/He mouse ventricles with coxsackievirus B3 (CVB3) and examined, by immunofluorescence, the expression of MHC class I and II antigens, previously reported not to be expressed by normal cardiac myocytes. Furthermore, to confirm the expression of MHC class I (H-2K\(^b\)) antigens at the cellular level, we treated cultured cardiac myocytes with interferon gamma and examined the antigen expression by immunofluorescence and Northern blot hybridization, using an antisense RNA probe for MHC messenger RNA. Our observations demonstrated 1) CVB3–induced myocarditis resulted in the enhanced expression of MHC class I (H-2K\(^b\)) gene product on the surface of cardiac myocytes but low or undetectable levels of MHC class II or H-2D\(^b\) gene products, and moderate focal transient (days 5–7) expression of both MHC class I (K\(^a\)+D\(^b\)) region gene products and MHC class II antigens were induced on capillary endothelial cells; 2) murine fetal cardiac myocytes cultured in vitro in the presence of interferon gamma similarly were shown to express marked levels of MHC class I (H-2K\(^b\)) but low to undetectable levels of the H-2D\(^b\) gene product; however, weak to moderate MHC Class II antigens were expressed by these cultured myocytes; and 3) the expression of MHC antigens in cardiac myocytes was modulated at the transcriptional level. These data strongly suggest that the expression of MHC class I antigens in cardiac myocytes induces the interaction between cardiac myocytes and T lymphocytes and that cytotoxic T lymphocytes in particular may play at least a partial role in the myocardial damage induced by viral infection. (Circulation Research 1990;67:360–367)

Viral myocarditis is a well-known disease associated with characteristic lesions such as necrosis of myofibers, inflammatory cell infiltration, and fibrosis. Because maximal inflamma-

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cardiac myocytes, there is little to no expression of MHC class I antigens and no expression of class II antigens. Recent reports have demonstrated a massive induction of donor-type MHC class I and II antigens in cardiac myocytes of allografts, indicating that cardiac myocytes may express both classes of MHC antigens. In acute viral myocarditis, persistent inflammation and destruction of cardiac myocytes suggest an interaction between cardiac myocytes and T lymphocytes. Virus-infected myocytes must express MHC antigens on their surfaces to become target cells for T lymphocytes. To our knowledge, no previous reports have demonstrated the expression of MHC antigens by cardiac myocytes in acute viral myocarditis.

The purposes of this study were, first, to examine in vivo the expression of MHC class I and II antigens in C3H/He mouse ventricular myocytes infected with coxsackievirus B3 (CVB3) by immunofluorescence, and second, to confirm that cardiac myocytes themselves express MHC antigens on their surfaces and that this expression is modulated at the transcriptional level. For the second purpose, we induced MHC antigens in cultured ventricular myocytes with interferon gamma (IFN-γ) and examined antigen expression by immunofluorescence and Northern blot hybridization, using an antisense RNA probe for MHC mRNA.

Materials and Methods

Animals

C3H/He male mice, 3–7 weeks old, were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan.

Monoclonal Antibodies

As anti-mouse MHC monoclonal antibodies (mAb) for class I antigens, anti-H-2Kα and anti-H-2Dβ were used; for class II antigens, anti-Iaβ was used (which can detect both I-Aβ and I-Eβ antigens). All were purchased from Meiji Institute of Health Science, Kanagawa, Japan. Anti-Lyt-2 and anti-L3T4 antibodies were obtained from the hybridomas 53–6.7 and GK1.5, respectively. The preparation of ant cardiac myosin mAb (CMA19) has been described previously. The reactivity of CMA19 for C3H/He mouse ventricular myosin heavy chain was confirmed by immunoblot analysis using biotinylated horse antimouse immunoglobulin G (IgG) antibody and horse radish peroxidase–avidin D (Vector Laboratories Inc., Burlingame, Calif.) (data not shown).

Virus

CVB3 (Nancy strain) and FL (human amnion) cells were kindly supplied by Dr. Y. Kitaura (Osaka Medical College, Osaka, Japan). CVB3 was grown in FL cells in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum. The virus preparations had a titer of 4×10⁶ plaque-forming units/ml and were stored at −70°C.

Infection of Mice

Seven-week-old mice were inoculated intraperitoneally with 2×10⁶ plaque-forming units of virus in 0.5 ml DMEM containing 10% fetal bovine serum. They were killed on days 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 21, 28, and 49.

Preparation of Cultured Cardiac Myocytes

Heart ventricles were aseptically removed from 14- to 16-day-old fetal mice, minced in calcium-free phosphate buffered saline (PBS), and digested with 0.06% trypsin-EDTA in PBS. The isolated cardiac myocytes were washed in DMEM containing 10% fetal bovine serum, dispersed in plastic dishes for 1 hour to separate from the fibroblasts, and then removed to culture flasks and tissue culture chamber slides (Miles Inc., Diagnostics Div., Kankakee, Ill.). They were cultured overnight at 37°C in a humidified 5% CO₂–95% air incubator and were then divided into two groups, designated A and B. The culture medium was replaced with DMEM containing insulin-transferrin-sodium selenite media supplement (Sigma Chemical Co., St. Louis, Mo.); then recombinant murine IFN-γ (Shionogi & Co., Ltd., Osaka, Japan) was added to group B to a concentration of 100 units/ml. The cardiac myocytes, after 48 hours and after 4 days under these conditions, were used for Northern blot hybridization and immunocytochemical study, respectively.

We also cultured the remaining fibroblasts for 1 week after separation from myocytes. They were divided into control and IFN-treated groups and were used for Northern blot hybridization after 48 hours of culture.

Immunohistochemical Study

The titers of anti-MHC mAb were tested using spleen tissue. Freshly dissected ventricles were frozen in OCT compound (Miles) in liquid nitrogen. Cryostat sections of 6 μm were prepared, air-dried, and fixed in acetone for 5 minutes. They were incubated with biotinylated or fluorescein isothiocyanate (FITC)–conjugated anti-MHC mAb at 37°C for 60 minutes, washed three times in PBS, then incubated with FITC-conjugated avidin D at 37°C for 30 minutes. The second incubation was not necessary for FITC-conjugated mAb. They were washed three times in PBS and were mounted on coverslips with glycerin. They were examined and photographed under a Nikon MICROPHOT-FX fluorescence microscope.

For in vitro study, to ensure that the cultured cells were cardiac myocytes, we performed double staining for cardiac myosin heavy chain as well as MHC antigens. The cultured cells on slides were washed in PBS and fixed in acetone for 5 minutes. They were incubated with CMA19 at 37°C for 60 minutes, washed three times in PBS, then incubated with phycoerythrin (PE)–conjugated anti-mouse IgG antibody at 37°C for 60 minutes, and washed again three times in PBS. The subsequent procedures for stain-
ing MHC class I (H-2K^a, H-2D^a) and class II (Ia^b) antigens were the same as for tissue samples.

For Northern blot hybridization analysis, to evaluate the effects of contamination by nonmuscle cells (most of which were fibroblasts), we measured their ratio to total cells by double staining for cardiac myosin heavy chain (labeled with PE) and H-2K^a antigen (labeled with FITC) and analyzing by flow cytometry (FACStar, Becton Dickinson Immunocytoometry Systems, Mountain View, Calif.). We found the rate of contamination to be 34% after 48 hours of culture (data not shown).

Preparation of Hybridization Probes

An H-2K^a DNA clone, a generous gift from Dr. J. Miyazaki (Institute for Medical Genetics, Medical School, Kumamoto University, Kumamoto, Japan), in which an EcoRI fragment of about 9 kilobases including the H-2K^a gene from a cosm id was subcloned into a pBR322 vector, has been described in detail.14 A BamHI fragment of about 2.3 kilobases from the insert DNA, which encodes the less polymorphic α and transmembrane domains, was subcloned into pBluescript SK(+) vector (Stratagene Inc., La Jolla, Calif.), then linearized by EcoRI to allow for synthesis of a ^32P-labeled antisense RNA probe. An antisense RNA probe (4.0×10^6 cpm/μg) was synthesized by T3 RNA polymerase using ^32P[UTP, an unlabeled mixture of ATP, GTP, CTP, dithiothreitol, and human placental ribonuclease inhibitor.

A mouse α-actin complementary DNA (cDNA) clone (plasmid 91), in which a Pst I fragment of about 1.3 kilobases of α-actin cDNA was sub cloned into a pBR322 vector, has been described in detail.15 A Pst I fragment of about 1.1 kilobases from the insert DNA, which represents approximately 90% of the coding sequence for α-actin, was subcloned into pBluescript SK(+) vector, then linearized by Xba I to allow for synthesis of a ^32P-labeled antisense RNA probe. An antisense RNA probe (2.3×10^6 cpm/μg) was synthesized by T7 RNA polymerase in the same way as the H-2K^a probe. This probe hybridizes with both muscle α-actin and nonmuscle β-actin messenger RNAs (mRNAs).

Northern Blot Hybridization

Total cytoplasmic RNA was prepared from cardiac myocytes and fibroblasts by a method previously described.16 In both the control and the IFN-treated groups, 20 μg of cytoplasmic RNA from cardiac myocytes and 6.8 μg from fibroblasts, which was considered to be the level of contamination, were subjected to formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. After it was dried in vacuum for 1 hour at 80°C, the nylon membrane was prehybridized in a solution containing salmon sperm DNA (200 μg/ml) for 4 hours at 65°C, then hybridized with the ^32P-labeled H-2K^a antisense RNA probe overnight at 65°C. After the nylon membrane was washed, it was autoradiographed for 4 hours at −70°C.

To confirm that equivalent amounts of RNA from the control and the IFN-treated groups were loaded onto the gel, hybridization with the α-actin probe was also performed in the same way.

Results

In Vivo Studies

Expression of major histocompatibility complex class I and II antigens in ventricular tissues. There was no staining of MHC class I (H-2K^a, H-2D^a) and class II (Ia^b) antigens in normal ventricular tissues, including sarcolemma, intercalated disks, and capillary endothelial cells (Figures 1A, 1E, and 1G). Until day 3 after virus inoculation, there was also no staining of MHC class I and II antigens and no cell infiltration. On days 4 to 5, clear expression of MHC class I (H-2K^a) antigen appeared on sarcolemma of myocytes, and massive cell infiltrations were also seen focally scattered over the myocardium. Nonuniform expression of H-2K^a antigen, which reached a maximum level around day 7 and continued for 3–4 weeks after virus inoculation, was seen over the myocardium and around the areas of cell infiltration (Figure 1B) or adjacent to them in serial sections (Figure 1C). However, low or undetectable levels of MHC class II (Ia^b) or H-2D^a antigen were seen on sarcolemma of myocytes throughout the course (Figures 1F and 1H). For capillary endothelial cells, moderate focal expression of both MHC class I (H-2K^a, H-2D^a) and class II (Ia^b) antigens was detected around day 7 (Figure 1D). The time course of the expression of MHC antigens in ventricular tissues is summarized in Table 1.

In Vitro Studies

Expression of major histocompatibility complex class I (H-2K^a) antigen in cultured ventricular myocytes. Figure 2 shows double-stained ventricular myocytes cultured in serum-free medium for 4 days. Figures 2A and 2B show the staining pattern specific for H-2K^a antigen. Figures 2C and 2D, which correspond
TABLE 1. Expression of Major Histocompatibility Complex Antigens in Ventricular Tissues Infected With Coxsackievirus B3

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Myocytes</th>
<th>Capillary endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class I</td>
<td>Class II</td>
</tr>
<tr>
<td></td>
<td>H-2Kβ</td>
<td>H-2Dα</td>
</tr>
<tr>
<td>1–3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5–7</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>+ + + +</td>
<td>–</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>28</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>49</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
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Intensity of staining shown as: -, negative; ±, weakly positive; +, moderately positive; ++, strongly positive; + + + +, strongly positive and moderately positive for H-2Kβ antigen distributed with variations.

to Figures 2A and 2B, respectively, show the staining pattern specific for cardiac myosin heavy chain and indicate that most of the cells are cardiac myocytes. There was very slight to no expression of H-2Kβ antigen in ventricular myocytes in the control group (Figure 2A). After treatment with IFN-γ, most of the ventricular myocytes strongly expressed H-2Kβ antigen on their surfaces (Figure 2B).

For H-2Dα and Iα4 antigens, after treatment with IFN-γ, very slight to no expression of H-2Dα antigen and weak to moderate expression of Iα4 antigen were induced in ventricular myocytes (data not shown).

Flow cytometric analysis of ventricular myocytes after 48 hours of culture revealed two populations differing in the levels of myosin heavy chain in both control and IFN-treated groups. Cells of populations 1 and 2 expressed lower and higher levels of myosin heavy chain and were considered to be fibroblasts and myocytes, respectively. Population 1 cells expressed moderate levels of H-2Kβ antigen, and their levels were slightly increased by IFN treatment. In contrast, population 2 cells expressed very low levels of H-2Kβ antigen, and their levels were markedly increased by IFN treatment (data not shown).

Modulation of expression of major histocompatibility complex mRNA in ventricular myocytes. The immunocytochemical study showed that ventricular myocytes could express MHC class I (H-2Kβ) antigen at levels that were significantly increased by treatment with IFN-γ. To confirm their expression at the transcriptional level, we performed Northern blot hybridization analysis using a 32P-labeled H-2Kβ antisense RNA probe, which may hybridize with all of the MHC class I– and class II–like transcripts.

Figure 3 shows the results of Northern blot hybridization analysis of the ventricular myocytes and the fibroblasts exposed to a serum-free medium alone or to a medium containing IFN-γ (100 units/ml) for 48 hours. Figure 3A shows the levels of actin mRNA both for ventricular myocytes (lanes 1 and 2) and fibroblasts (lanes 3 and 4) as internal standard. This confirmed that equivalent amounts of RNA were prepared from the control and the IFN-treated groups of both the ventricular myocytes and the fibroblasts. Figure 3B shows the levels of MHC mRNA. The bands observed in lanes 3 and 4 show the levels of contamination by fibroblasts in the control and the IFN-treated groups, respectively. Undetectable levels of MHC mRNA were found in the control group (lane 3), and significant levels were induced by treatment with IFN-γ (lane 4). Even considering the levels of contamination, ventricular myocytes in the control group expressed only low levels of MHC mRNA (lane 1), and those levels were markedly increased by treatment with IFN-γ (lane 2).

Discussion

In this study, we have clearly demonstrated that in acute viral myocarditis caused by CVB3, MHC class I (H-2Kβ) antigens were strongly induced in cardiac myocytes, whereas low or undetectable levels of MHC class II (Iα4) or H-2Dα antigens were observed. We used splenic tissue samples as positive controls and confirmed that the potential of the mAbs to recognize the determinants of H-2Dα and Iα4 antigens could not be modified with acetone fixation (data not shown). However, we could not exclude the possibility that there was a low level of expression of H-2Dα antigen below the limit of sensitivity of the immunofluorescence technique.

Recent studies7,17 have revealed that a few types of nucleated cells express only weak or undetectable levels of MHC class I antigens. These cells include endocrine cells in the thyroid, parathyroid, and pituitary glands, and pancreas as well as spermatozoa, Leidig cells, fetal amnion epithelial cells, and fetal trophoblasts, which are related to the reproduction process. They also include neurons in the brain and skeletal and cardiac myocytes. The suppression of MHC antigens can be considered a means of protecting these cells against T cell–mediated immunological surveillance. It has been recently demonstrated that in some of these cells, such as neurons and amion epithelial cells, the expression of MHC class I antigens can be induced by IFN.18–21 This indicates that the expression of MHC antigens in these cells is under regulatory control rather than being genetically defected. Many human tumors, such as small cell lung carcinoma and neuroblastoma, are known to express greatly reduced levels of MHC class I antigens.22 The suppression of class I antigens in these cells, which enables them to escape immune detection, appears to correlate with their rapid growth and metastasis. Ono et al23 showed that MHC class I antigens were strongly expressed in pancreatic islet cells of newly diabetic Bio-Breeding rat, which is an animal model of spontaneous insulin-dependent diabetes mellitus. These authors demonstrated that inflammatory infiltrations were always present adjacent to islet cells exhibiting enhanced class I antigen expression. They further suggested that inflammatory cells might induce the enhanced expression of class I
antigens in islet cells via cytokines and facilitate the recognition of target cells by CTL.

For cardiac myocytes, several studies9-11 on transplanted cardiac muscles showed that high levels of MHC class I and II antigens were expressed in cardiac myocytes and endothelial cells just before episodes of histological rejection. Our data strongly suggest that MHC class I (H-2K^k) antigens, which were predominantly induced in cardiac myocytes around cell infiltrations shortly after infection by the release of cytokines or by unknown mechanisms, facilitated the interaction between CVB3-infected cardiac myocytes and CTL. The involvement of CTL is also suggested by the fact that the CTL subpopulation in spleen decreased significantly just after the induction of H-2K^k antigen (data not shown). Recently, Kishimoto et al.24 showed that Lyt-2^+ cells, rather than L3T4^+ cells or B cells, predominantly infiltrated the hearts with viral myocarditis, suggesting the possible involvement of CTL at the site of inflammation.

Our present study revealed the disparity between expression of H-2K^k and H-2D^b antigens in cardiac myocytes of acute viral myocarditis. Many studies25 have shown that virus-specific, syngeneically restricted CTLs are monospecific for both viral antigens and restricting H-2K or H-2D antigens; that is, they operate only in association with either H-2K or H-2D antigens, but not with both.26 The monospecificity of the restriction by H-2K or H-2D antigen was also evidenced by antibody blocking studies.27 However, the real mechanism of disparity between enhancement of H-2K and H-2D genes is unknown. Several studies have investigated the immune protection against viral growth by adoptive transfer of lymphocytes. Zinkernagel and Welsh28 showed that in vivo protective effects depended completely on immune T cells compatible with H-2K or H-2D antigen, and that Ia antigen-compatible T cells did not confer a significant level of protection. These studies strongly suggested that virus-specific CTLs, which were restricted syngeneically by H-2K or H-2D antigen but not by Ia antigen, lysed the virus-infected target cells in vivo as well as in vitro and played major roles in preventing viral growth. For CVB3-induced myocarditis in BALB/c mice, in vivo studies of both adoptive transfer of immune T cells and antibody treatment strongly suggested that Lyt-2^+ CTLs, which were independent of interleukin-2, were predominantly involved in this disease.4,29 This was also supported by the fact that
cyclosporin-A, which suppresses interleukin-2 production by L3T4+ Th, failed to prevent CVB3-induced myocarditis.29-31 Our data in the present study also suggest that CTLs may play an important role in CVB3-induced acute myocarditis, because their operation is restricted by MHC class I antigens.

Recent studies19,20,32,33 have shown that IFN increased or induced the expression of MHC class I or II antigens in lymphoid cells, neuronal cells, and amnion cells. In the present study, we also showed that the low level of expression of MHC antigens in cardiac myocytes could be enhanced by IFN-γ. These results indicate that failure to express MHC antigens in neuronal and amnion cells, as well as in cardiac myocytes, is not due to genetic defects but to down-regulation at the transcriptional level. One of the reasons for such down-regulation may be to protect damaged or degenerated cells in these tissues against T cell–mediated immunological surveillance. To elucidate the primary mechanism for enhancement of MHC antigen expression in acute viral myocarditis, further investigation is required.

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**KEY WORDS** • coxsackievirus B3 • viral myocarditis • cytotoxic T lymphocyte • interferon gamma • major histocompatibility complex
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