American Journal of Pathology, Vol. 150, No. 6, June 1997

Intracellular Viral Localization in Murine Coxsackievirus-B3 Myocarditis

Ultrastructural Study by Electron Microscopic in Situ *Hybridization*

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Group B Coxsackieviruses are a common cause of myocarditis. To detect the viral genome and its localization in the myocardium, we examined C3H/He mice with Coxsackievirus B3 (CVB3) myocarditis on days 5, 8, and 14 after inoculation by the reverse transcriptase polymerase chain reaction and by* in situ *hybridization. Sense and antisense CVB3 RNA were detected in the myocardium of all mice up to day 14 by reverse transcriptase polymerase chain reaction. Light microscopic* in situ *hybridization with a cDNA probe for CVB3 showed clusters of positive signals in the areas of myocardial necrosis and cell infiltration. With electron microscopic* in situ *hybridization, CVB3 RNA was detected in the cytoplasm of cardiocytes, between the myofibrils, near the mitochondria, and in tubular or vesicular structures. Viral RNA was also detected in necrotic debris, in the cytoplasm of macrophages, and in the cytoplasm of interstitial fibroblasts. These findings suggest that CVB3 RNA is replicated in the cytoplasm of cardiocytes, transferred into tubular or vesicular structures, released into the interstitium, and phagocytosed by macrophages. Some positive signals were also detected in the cytoplasm of cardiocytes showing close contact with infiltrating lymphocytes, suggesting that the lymphocytes recognized virus-infected cardiocytes and caused cell-mediated immune cardiocyte damage. (Am J Pathol 1997, 150:2061–2074)

Group B Coxsackieviruses are considered to be the most common cause of viral myocarditis in both humans and animals.[1] Coxsackieviruses not only cause myocarditis but may also be responsible for dilated cardiomyopathy.[2–11] However, the details of the infectious cycle of these viruses in the myocardium and the mechanism of cardiocyte injury are not well clarified.

The infectious cycle of picornaviruses in host cells is generally considered to be as follows. Picornaviruses are single-plus-strand RNA viruses that enter the target cell and uncoat the capsid protein, thus releasing plus RNA (sense RNA). The virus is then replicated via antisense RNA transcribed by a virus-specified RNA-dependent RNA polymerase.[10–15] The antisense RNA intermediately serves as a template for the synthesis of new sense RNA. Finally, cell lysis and virus release generally complete the cycle of acute enteroviral infection.

In situ hybridization can localize target sequences within a specific tissue, unlike filter hybridization methods, and can be applied to detect specific nucleic acids within tissue sections.[3,5,6,13–18] Recently, nonradioactive probes have been developed that provide a high spatial resolution and precise localization, leading to the application of *in situ* hybridiza-

Supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, and Culture and by a Research Grant for Intractable Diseases from the Japanese Ministry of Health and Welfare.

Accepted for publication March 20, 1997.

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tion combined with electron microscopy.[18–21] The intracellular distribution of poliovirus RNA in cultured cells infected with the virus was previously determined in this way.[21]

However, the infection cycle in the myocardium of mice with Coxsackievirus B3 (CVB3) myocarditis has not been clarified *in vivo*. Accordingly, we investigated the presence and localization of the CVB3 genome in mice with myocarditis, using reverse transcriptase polymerase chain reaction (RT-PCR) gene amplification of viral sense and antisense RNA as well as *in situ* hybridization with examination by light and electron microscopy.

Materials and Methods

Viruses, Animals, and Infection

The Nancy strain of CVB3 was serially propagated in monolayer cultures of FL cells in Eagle's medium with 1% calf serum and was harvested by several consecutive freezing and thawing cycles after the cytopathic effect developed. The fluid was pooled and centrifuged at 10,000 rpm for 15 minutes, after which the supernatant was stored at −20°C until inoculation.

Male C3H/He mice (3 weeks old) were purchased from Japan SLC, (Shizuoka, Japan). Fifteen animals were inoculated with the Nancy strain of CVB3 (0.5 ml of 10^7 $TCID_{50}$/ml) and killed on days 5, 8, and 14 after inoculation. As controls, five mice were inoculated with 0.5 ml of the supernatant of virus-free FL cell culture medium and were sacrificed on day 5.

Processing of Myocardial Tissue

After the mice were killed, their hearts were excised and the ventricles were divided into four blocks, with one each being for virus isolation, RT-PCR, light microscopic study (*in situ* hybridization and light microscopic immunohistochemical

a)

oligonucleotide	location	sequence(5'→3')
primer A	3,202-3,221	GACTCTGCCAATACGAGAAG
primer B	3,289-3,270	TTTGTCATTGTAGTGATGCT
probe	3,267-3,243	GCCTAGTAGTGGTAACTCCGCTGGG

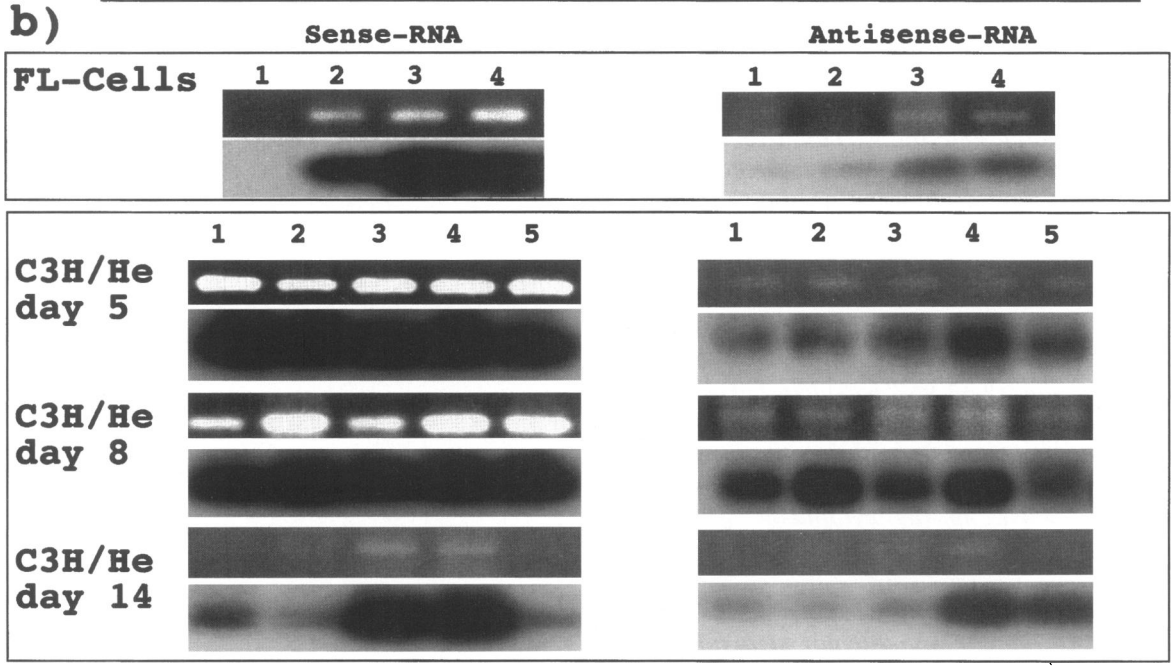

Figure 1. *RT-PCR study.* a: *The oligonucleotides synthesized for primers A and B and the probe. The target of PCR gene amplification was the 88-bp sequence corresponding to the VP-1 region of the virus. The base locations are numbered from their published sequences.* b: *Strand-specific RT-PCR detection of sense RNA and antisense RNA for CVB3 in FL cells and in the hearts of CVB3-inoculated C3H/He mice.* Upper panels: *Agarose electrophoresis of PCR products.* Lane 1, *uninfected FL cells;* lanes 2 *to* 4, *CVB3-infected FL cells at 1, 4, and 12 hours after infection, respectively.* Lower panels: *Southern blot hybridization of the electrophoresed gel probed with a* ^{32}*P-labeled internal oligonucleotide.* Lanes 1 to 5, *myocardium from CVB3-infected C3H/He mice (mice 1 to 5, respectively) on day 5, 8, and 14, as indicated. See text.*

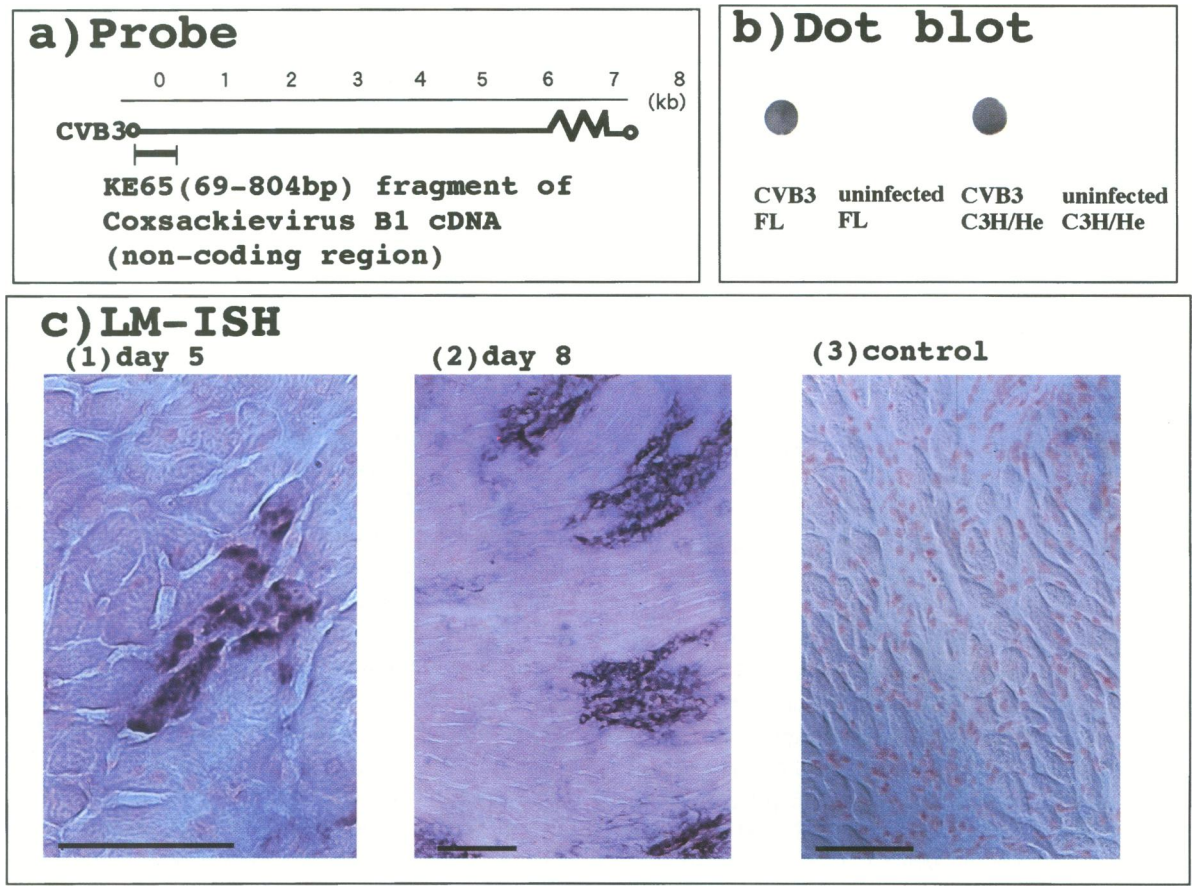

Figure 2. *The probe and light microscopic* in situ hybridization. **a:** *Diagram of the CVB3 genome and the KE65 fragment of CVB1 cDNA (69 to 804 bp), which shows the greatest homology between CVB1 and CVB3.* **b:** *Detection of the CVB3 genome in CVB3-infected FL cells (CVB3FL), uninfected FL cells (FL), CVB3-infected mouse hearts (CVB3 C3H/He), and uninfected mouse hearts (C3H/He) by dot blot hybridization. CVB3-RNA could be detected in infected mouse hearts and infected FL cells, whereas no cross-hybridization was seen with the RNA of uninfected FL cells or uninfected myocardium.* **c:** *Light microscopic* in situ *hybridization (LM-ISH) detection of the CVB3 genome in the myocardium of CVB3-infected mice using a digoxigenin-labeled CVB1 cDNA probe. Bars, 50 μm.* **Panel 1:** *Ventricular myocardium from a C3H/He mouse on day 5. Positive signals (purple staining) were detected in the cytoplasm of some cardiocytes.* **Panel 2:** *Ventricular myocardium from a C3H/He mouse on day 8. Positive signals were detected in the cytoplasm of cardiocytes and inflammatory cells in the necrotic and inflammatory foci.* **Panel 3:** *Ventricular myocardium of a C3H/He mouse on day 8 hybridized with the pBR328 probe. No signals were detected.*

study), and electron microscopic *in situ* hybridization, respectively.

For virus isolation, one block of heart tissue was homogenized individually in 10 vol of Eagle's medium and centrifuged at 10,000 rpm for 30 minutes, and 0.1-ml aliquots of the supernatant were inoculated into culture tubes containing FL cells. The cytopathic effect was observed up to day 8 after inoculation, and $TCID_{50}$ was calculated.[22]

For RT-PCR, one block of heart tissue was homogenized with a Polytron homogenizer in 1 ml of RNA lysis buffer (Applied Biosystems, Foster City, CA), the constituents of which were not reported by the manufacturer except for 4 mol/L guanidium thiocyanate. Automated extraction of total RNA from the homogenate was performed using the ABI-340A Nucleic Acid Extractor (Applied Biosystems), according to the standard instructions supplied by the manufacturer.[3,23]

For light microscopic *in situ* hybridization and immunohistochemical studies, a block of heart tissue was placed in OCT compound (Tissue Tek II, Miles Laboratories, Elkhart, IN), quick-frozen in a liquid nitrogen bath, and stored at −80°C until processing.[3]

For electron microscopic *in situ* hybridization, a block of heart tissue was fixed for 45 minutes with 4.5% paraformaldehyde and 0.5% glutaraldehyde and then embedded in LR White (London Resin Company) as described by Timms.[24] The tissue was placed in the bottom of gelatin capsules (Lilly Pharmaceuticals, Indianapolis, IN), and ultrathin sections were cut with a diamond knife on an ultramicrotome (2088ULTROTOME, LKB), retrieved on 200-mesh nickel grids, and stored until use.

Table 1. *Virus Titers in the Murine Hearts of Coxsackievirus-B3-Inoculated Mice*

Days after inoculation	Virus titer of heart homogenate*				
5	4.0	5.5	6.0	6.0	6.5
8	3.5	5.0	5.5	6.0	6.0
14	–	–	–	–	–

*Expressed as $\log_{10}$[$TCID_{50}$/g (wet weight of cardiac tissue)]. –, <0.1.

RT-PCR Gene Amplification

A pair of oligonucleotide primers and a probe were synthesized by Takara Shuzo Co. (Kyoto, Japan) based on the published nucleotide sequences of the CVB3[25] genome (Figure 1a). For Southern blot hybridization, the probe was labeled with ^{32}P-labeled ATP (5000 cpm/mmol/L, Amersham International, Little Chalfont, UK) using a Megalabel 5′ end-labeling kit (Takara Shuzo).

One-half of the RNA extracted from FL cells or mouse hearts was used as the initial RNA template for the virus-specific first sense or antisense cDNA strand. A reverse transcriptase reaction to detect sense or antisense RNA was carried out at 42°C for 60 minutes in a reaction mixture (25 μl) containing the following: 50 mmol/L Tris/HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L 1,4-dithiothreitol, 0.6 mmol/L each dATP, dGTP, dCTP, and dTTP, 0.4 μmol/L antisense primer B or sense primer A, 50 U of human placental RNAse inhibitor, and 500 U of cloned Moloney MuLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). Subsequently, the first-strand reaction mixture was diluted with an equal volume of PCR dilution buffer containing 25 mmol/L KCl, 0.8 μmol/L sense primer A with 0.4 μmol/L antisense primer B (or 0.8 μmol/L antisense primer B with 0.4 μmol/L sense primer A), 0.02% (w/v) gelatin, and 1.25 U of Ampli*Taq* DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT). Amplification was performed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 15 seconds (plus 1 second in each new cycle), and 10-μl aliquots of each PCR product were analyzed by electrophoresis on 3% Nuseive/1% Seakem agarose gel (FMC Bioproducts, Rockland), followed by ethidium bromide staining (Figure 1b). Next, the products were further analyzed by Southern blot hybridization with the ^{32}P-labeled probe (Figure 1b). All Southern blot hybridization procedures were performed according to the standard manufacturer's protocol for the HiBond-N+ nylon membrane (Amersham International). Autoradiography was performed for 48 hours at −80°C. We have previously reported on the sensitivity and specificity of our amplification procedure for the 88-bp sequence of CVB3 cDNA.[3,23] The 88-bp sequence was identified in samples with a viral content of less than 10^0 $TCID_{50}$ by ethidium bromide staining, whereas Southern blot hybridization revealed it in samples with a content of 10^{-1} $TCID_{50}$.

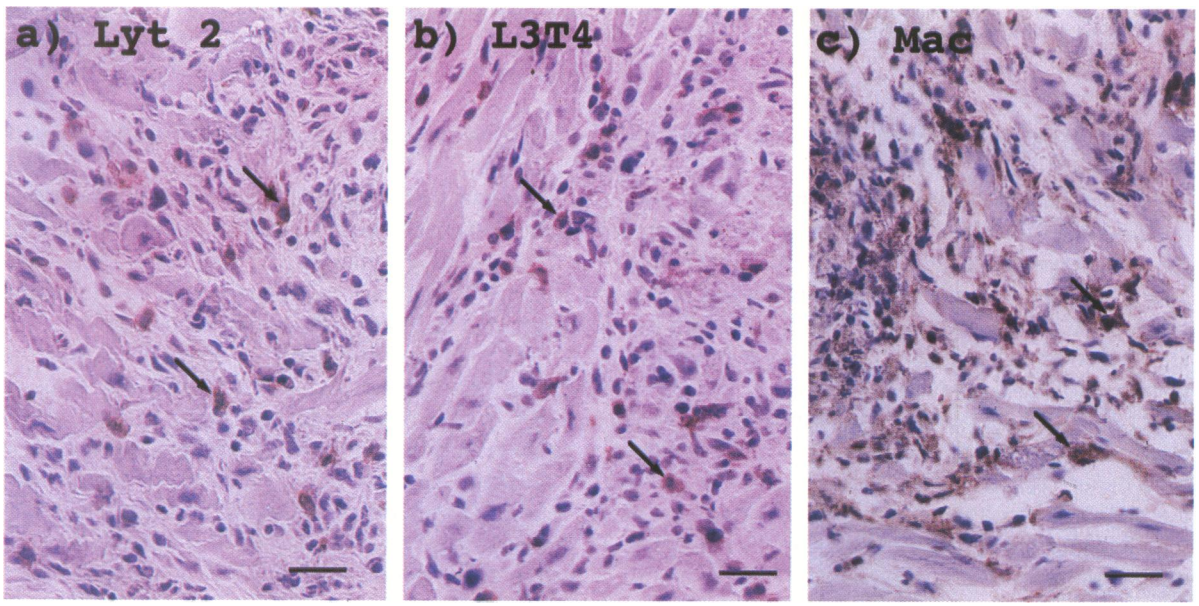

Figure 3. *Light microscopic immunohistochemistry of the myocardium of mice with CVB3 myocarditis on day 8. The distribution of Lyt 2 cells* (a, *L3T4 cells* (b), *and macrophages* (c) *was identified by the red-brown reaction products on the cell membranes. Bar, 20 μm.*

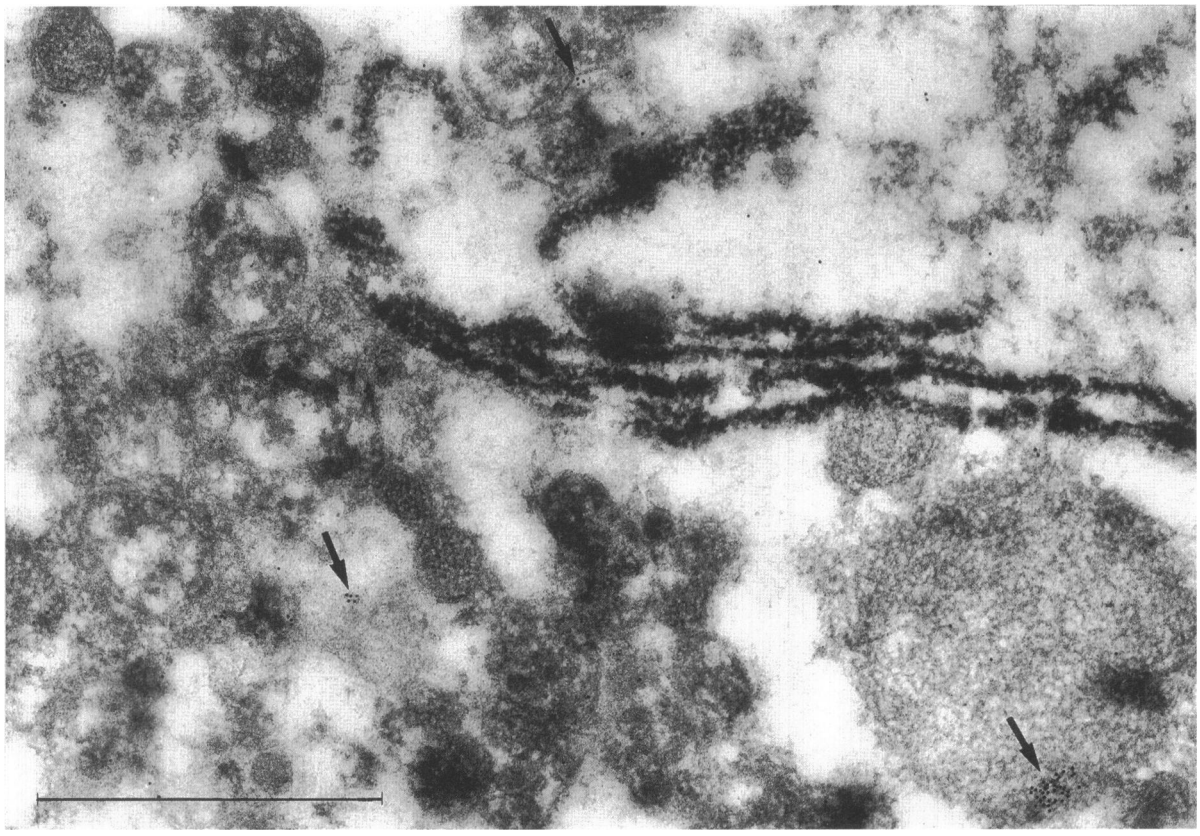

Figure 4. *Immunogold electron micrograph of an FL cell infected with CVB3 at 12 hours after virus inoculation. The digoxigenin-labeled cDNA probe and gold-labeled anti-digoxigenin antibodies reveal the distribution of CVB3 RNA. Hybridization signals* (arrows) *were detected in and around the amorphous structures in the degenerated cytoplasm. Bar, 1 μm.*

Dot-Blot Hybridization

The KE65 fragment (69 to 804 bp) of Coxsackievirus B1 (CVB1) cDNA[26] (a kind gift from Dr. A. Nomoto, Tokyo University, Tokyo, Japan), which is the region showing the greatest homology with CVB3, was labeled with digoxigenin-dUTP (Boehringer Mannheim, Mannheim, Germany) using hexanucleotides and Klenow enzyme (Boehringer[3,27]; Figure 2a). The specificity of this probe was confirmed by dot-blot hybridization.

Total RNA was extracted from CVB3-infected and uninfected FL cells, the hearts of mice with CVB3 myocarditis, and uninfected hearts, and 1 μg of RNA was denatured at 65°C for 5 minutes, spotted onto a HiBond-N+ nylon filter (Amersham International), and fixed in 0.04 mol/L NaOH. Next, the samples on the filters were incubated at 42°C for 14 hours in a heat-sealed bag with the hybridization solution (50%(v/v) formamide, 6X standard saline citrate (SSC), 5X Denhardt's solution, 0.5% (w/v) sodium dodecyl sulfate (SDS), and 30 ng/ml digoxigenin-labeled CVB1 cDNA probe). After hybridization, the filters were washed three times with 2X SSC and incubated twice for 15 minutes at 65°C in 2X SSC with 0.5% SDS, once at 65°C in 1X SSC with 0.5% SDS, and twice for 10 minutes at 65°C in 0.1X SSC with 0.5% SDS. The hybridized probes were detected colorimetrically using an alkaline-phosphatase-conjugated antibody specific for digoxigenin. Signals for the CVB1 cDNA probe were detected in RNA extracted from CVB3-infected FL cells and CVB3-infected mouse hearts but not in RNA from uninfected FL cells and uninfected hearts (Figure 2b).

This cDNA probe was also used for *in situ* hybridization, and pBR328 DNA labeled with digoxigenin-dUTP was used as the negative control.

Light Microscopic Immunohistochemical Study

Frozen sections of heart tissue (6 μm thick) were fixed with 4% paraformaldehyde on microscopic slides coated with Denhardt's solution. Infiltrating cell subsets were identified by immunohistochemistry with antibodies against specific antigens of cyto-

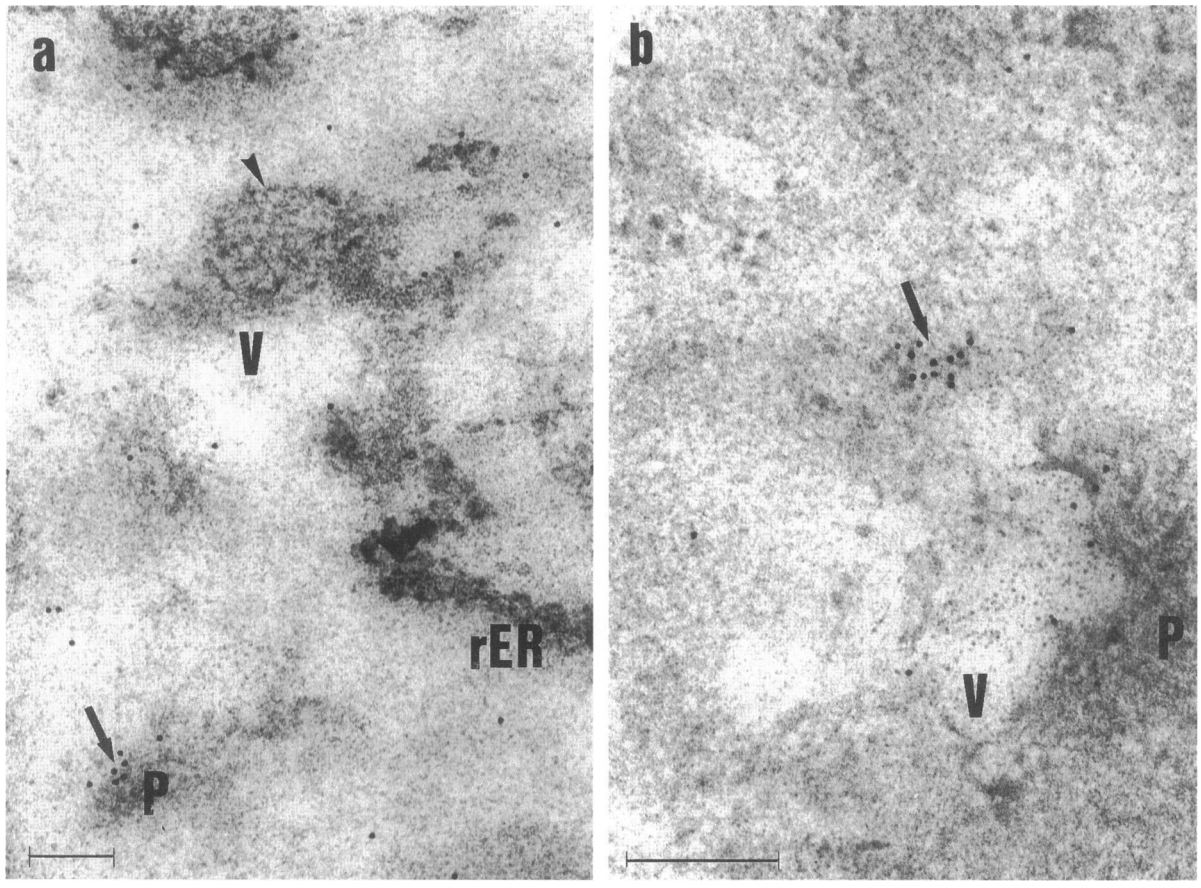

Figure 5. a: *Immunogold electron micrograph of an FL cell infected with CVB3 at 12 hours after virus inoculation. Electron-dense products (P), a virus-like particle* (arrowhead), *and translucent vesicles (V) are associated with rER. Positive signals* (arrow) *are seen on the electron-dense products. Bar, 0.5 μm.* b: *Immunogold electron micrograph of an FL cell infected with CVB3 at 12 hours after virus inoculation. Positive signals* (arrow) *are found on the electron-dense products (P) close to the translucent vesicles (V). Bar, 0.5 μm.*

toxic/suppressor T cells (Lyt 2), helper T cells (L3T4; a kind gift of Dr. T. Shirai, Juntendou University, Tokyo, Japan), and macrophages (Biosys, S.A.). Signals were detected with an ABC kit (Vectastain, Vector Laboratories, Burlingame, CA). The sections were observed by light microscopy after staining with hematoxylin.

Light Microscopic in Situ *Hybridization*

Frozen sections of heart tissue (6 μm thick) were fixed with acetic acid/ethanol (3:1) on microscopic slides coated with Denhardt's solution. After immersion in 0.2 N HCl for 20 minutes at room temperature and digestion with proteinase K (10 μg/ml for 15 minutes at 37°C), the samples were refixed with 4% paraformaldehyde for 5 minutes at room temperature, and hybridization was carried out in a mixture of 50% (v/v) formamide, 3X SSC, 1X Denhardt's solution, 10% dextran sulfate, 50 μg/ml denatured salmon sperm DNA, 100 μg/ml yeast RNA, and 1 μg/ml of the probe for 15 hours at 37°C in a humidified atmosphere. The sections were washed three times in 2X SSC for 1 hour at 37°C, twice in 1X SSC for 1 hour at 37°C, and twice in 0.5X SSC for 30 minutes at 37°C. The probe was detected colorimetrically using an alkaline-phosphatase-conjugated antibody specific for digoxigenin. Sections were observed by light microscopy after staining with nuclear fast red. Uninfected hearts and uninfected FL cells hybridized with the cDNA probe as well as infected sections hybridized with the pBR328 probe were used as controls.

Electron Microscopic in Situ *Hybridization*

In situ hybridization was performed with ultrathin sections on grids that were floated on droplets of the reaction mixture placed on Parafilm, with care being taken not to wet the reverse side of the grid. The sections were treated with proteinase K (0.1 μg/ml for 15 minutes at 37°C) and refixed in 4%

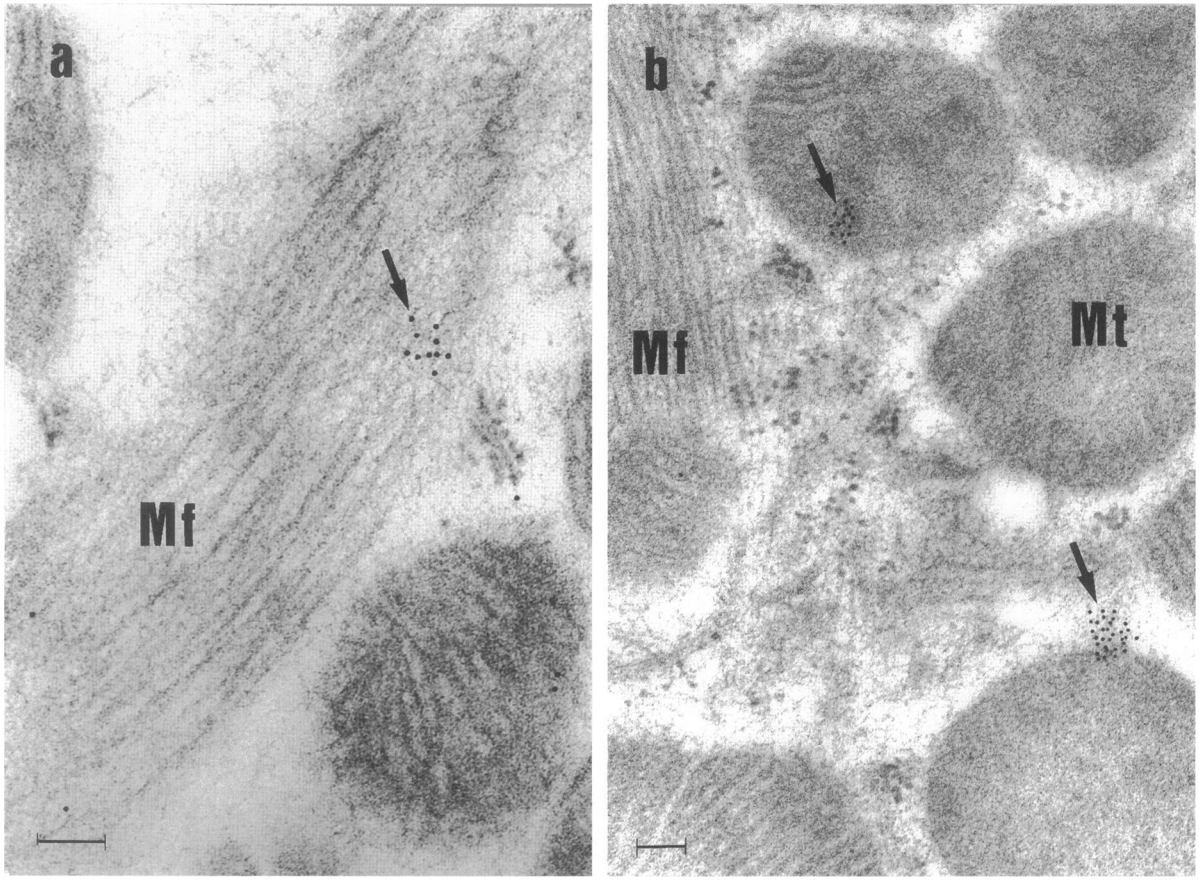

Figure 6. a: *Immunogold electron micrograph of a cardiocyte from a mouse with CVB3 myocarditis on day 5 after virus inoculation. Gold particles* (arrow) *representing the viral genome are seen in the myofibril of a cardiocyte. Bar, 0.1 μm.* b: *Immunogold electron micrograph of a cardiocyte from a mouse with CVB3 myocarditis on day 8 after virus inoculation. Two clusters of positive signals* (arrows) *are located near the mitochondria* (*Mt*). *Mf, myofibril. Bar, 0.1 μm.*

paraformaldehyde for 5 minutes at room temperature. After washing with 2X SSC, hybridization was carried out for 15 hours in 50% (v/v) formamide, 3X SSC, 1X Denhardt's solution, 10% dextran sulfate, 50 μg/ml denatured salmon sperm DNA, 100 μg/ml yeast RNA, and 1 μg/ml of the probe at 37, 45, 55, and 65°C in a humidified atmosphere. Next, the ultrathin sections were washed three times in 2X SSC for 1 hour at 37°C, twice in 1X SSC for 1 hour at 37°C, and twice in 0.5X SSC for 30 minutes at 37°C. Then the sections were incubated with antidigoxigenin-immunogold for 1 hour at 37°C, followed by staining with uranyl acetate and lead citrate before electron microscopy. Uninfected hearts and uninfected FL cells hybridized with the cDNA probe as well as infected sections hybridized with the pBR328 probe were used as the controls. In this and our previous studies,[28,29] we identified macrophages and lymphocytes by observing various morphological characteristics, including the nuclear/cytoplasmic ratio, phagocytotic vacuoles, and pseudopods, as well as assessing surface markers.

Electron Microscopic in Situ *Hybridization of Virus-Infected FL Cells as a Positive Control*

The temperature dependence of hybridization was tested by performing the procedure at 37, 45, 55, and 65°C, respectively. Background labeling decreased in proportion to the hybridization temperature, but too high a temperature caused structural deterioration, so 55°C was chosen for this study. On electron microscopic *in situ* hybridization, some FL cells showed a cytopathic effect characterized by destruction of the cell membrane, loss of the nuclei, and degeneration of the mitochondria. Gold particles representing CVB3 RNA were most often found in clusters of variable size. An analysis of cluster size showed that three or more particles constituted a positive signal. Gold particles were detected in and

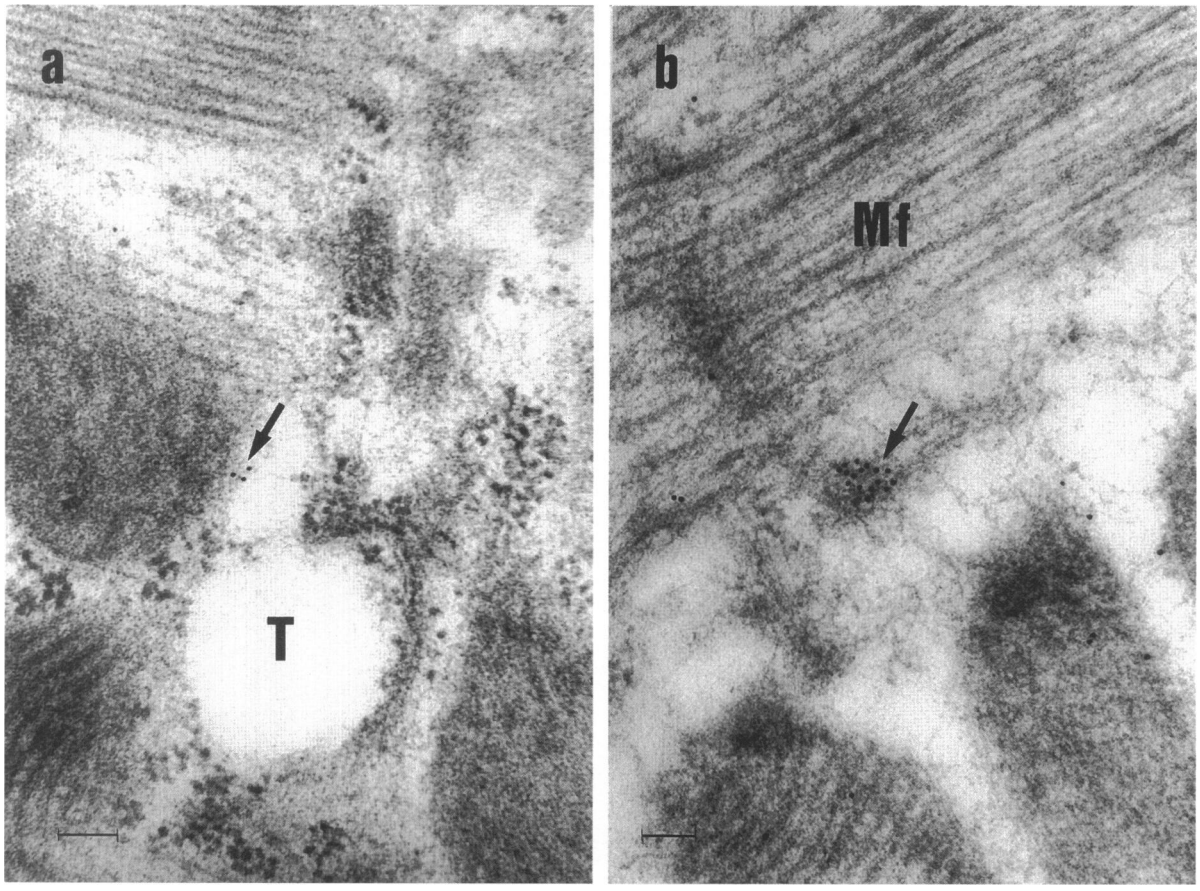

Figure 7. *Immunogold electron micrographs of cardiocytes in mice with CVB3 myocarditis.* a: *A cluster of hybridization signals* (arrow) *is observed in a small tubular structure (T) from a cardiocyte on day 5 after virus inoculation. The tubular structure is probably a T tubule.* b: *An aggregate of gold particles* (arrow) *is seen in close association with an apparently corbular sarcoplasmic reticulum near the Z band level of a myofibril on day 8 after inoculation. Mf, myofibril; Bars, 0.1 μm.*

around amorphous structures in the degenerated cytoplasm of CVB3-infected FL cells at 12 hours after inoculation (see Figure 4).

Results

Virological Study

All of the mice inoculated with CVB3 survived until the time of sacrifice. CVB3 was isolated from the myocardium of all mice on days 5 and 8 but was not detected on day 14 (Table 1).

In the RT-PCR study, both sense and antisense CVB3 RNA was detected in infected FL cells by Southern blot hybridization at 1, 4, and 12 hours after inoculation, although the antisense RNA signal was weak at 1 hour after inoculation (Figure 1b). Both sense and antisense CVB3 RNA were also found in the myocardium of all mice up to day 14 after inoculation by Southern blot hybridization, although the RNA level was decreased on day 14 (Figure 1b). The amplified product was not detected in samples from control hearts by either ethidium bromide staining or Southern blot hybridization (not shown). Sense RNA was detected up to day 28 after inoculation in our previous study.[3,23]

Histological Study

Light Microscopic Immunohistochemistry

The conventional and immunohistochemical light microscopic features of the myocardium were similar to those described in our previous papers.[11,28–31] On day 5, multiple necrotic foci were observed in the myocardium. On day 8, the necrotic areas were more prominent, and there were many lymphocytes (Figure 3, a and b) and macrophages (Figure 3c) in and around the necrotic foci. On day 14, the necrotic areas were smaller than on days 5 or 8, and fibrosis was noted in the interstitium.

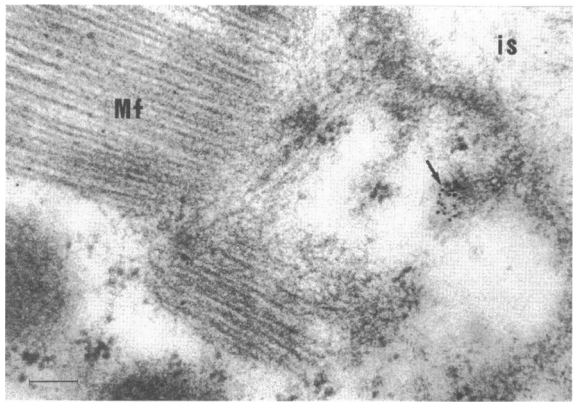

Figure 8. *Immunogold electron micrograph of a cardiocyte from a mouse with CVB3 myocarditis on day 5 after virus inoculation. Aggregation of many hybridization signals* (arrow) *is seen in a small vesicular structure closely associated with the plasma membrane. is, interstitial space; Mf, myofibril. Bar, 0.1 μm.*

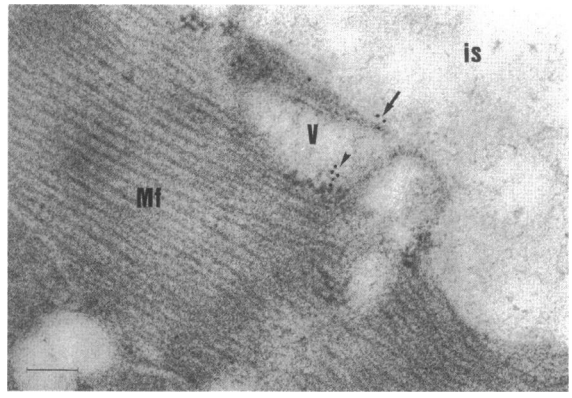

Figure 9. *Immunogold electron micrograph of a cardiocyte from a mouse with CVB3 myocarditis on day 8 after virus inoculation. A small vesicular structure (V) is apparently open to the interstitial space (is). One cluster of signals* (arrowhead) *appears to be located in the membrane of the vesicular structure, whereas the other* (arrow) *is closely associated with the plasma membrane. Mf, myofibril. Bar, 0.1 μm.*

Light Microscopic in Situ *Hybridization*

On days 5 and 8, positive hybridization signals were found in the necrotic and inflammatory foci, which were multiple and arranged randomly. Signals were also observed in the cytoplasm of degenerated and apparently viable cardiocytes. The greatest number of signals was noted on day 8 (Figure 2, c1 and c2). On day 14, positive signals were found in cardiocytes from the necrotic foci and in interstitial cells, including macrophages and fibroblasts, but the signals were less prominent than on days 5 and 8 (not shown). There were no signals in the myocardium on days 5, 8, and 14 when plasmid pBR328 DNA was used as the control probe (Figure 2c3).

Electron Microscopic in Situ *Hybridization*

On electron microscopic *in situ* hybridization, some FL cells showed a cytopathic effect in the *in vitro* study, and positive signals (clusters of three or more gold particles) were detected in and around amorphous structures in the degenerated cytoplasm of CVB3-infected FL cells (Figure 4). Electron-dense products, virus-like particles, and translucent vesicles were found in the vicinity of the rough endoplasmic reticulum (rER). Positive signals representing viral RNA were found on the electron-dense products associated with the rER (Figure 5, a and b).

Positive signals representing viral RNA were detected in the cytoplasm of necrotic, degenerated, or apparently intact cardiocytes on days 5 and 8 of the *in vivo* study. Immunogold particles were detected in the myofibrils, near the mitochondria (Figure 6), in association with the vesicular or tubular structures (Figures 7 to 9), and with the rER (data not shown) in the cytoplasm. Small vesicular and tubular membranous structures appeared to be sarcoplasmic reticulum or T-tubules. Some of them were closely associated with the plasma membrane (Figure 8), and some other vesicular structures were apparently open to the interstitium (Figure 9).

On days 5 and 8, some infiltrating lymphocytes in the necrotic foci were in close contact with the plasma membranes of degenerated or apparently intact cardiocytes, and gold particles were sometimes found in the cytoplasm near the points of contact (Figure 10). A number of macrophages were infiltrating the foci and phagocytosing necrotic debris on days 8 and 14 (Figure 11). The pseudopod-like cytoplasmic processes of these macrophages were often in contact with these necrotic debris, and gold particles were found in the debris and in the cytoplasmic processes of the macrophages. Clusters of virus-like particles were seen near the phagocytotic vacuoles in the cytoplasm of the macrophages, and gold particles were detected in and around the virus-like particles (not shown). On day 14, many fibroblasts had infiltrated the interstitium, and positive signals were also observed in the cytoplasm of cardiocytes (Figure 12) as well as fibroblasts. The signals in the cytoplasm of cardiocytes were weaker than those seen on days 5 and 8.

There were no signals in the myocardium on days 5, 8, and 14 when plasmid pBR328 DNA was used as the control. In addition, no signals were observed in myocardium obtained from uninfected control mice using the CVB1 cDNA probe (not shown).

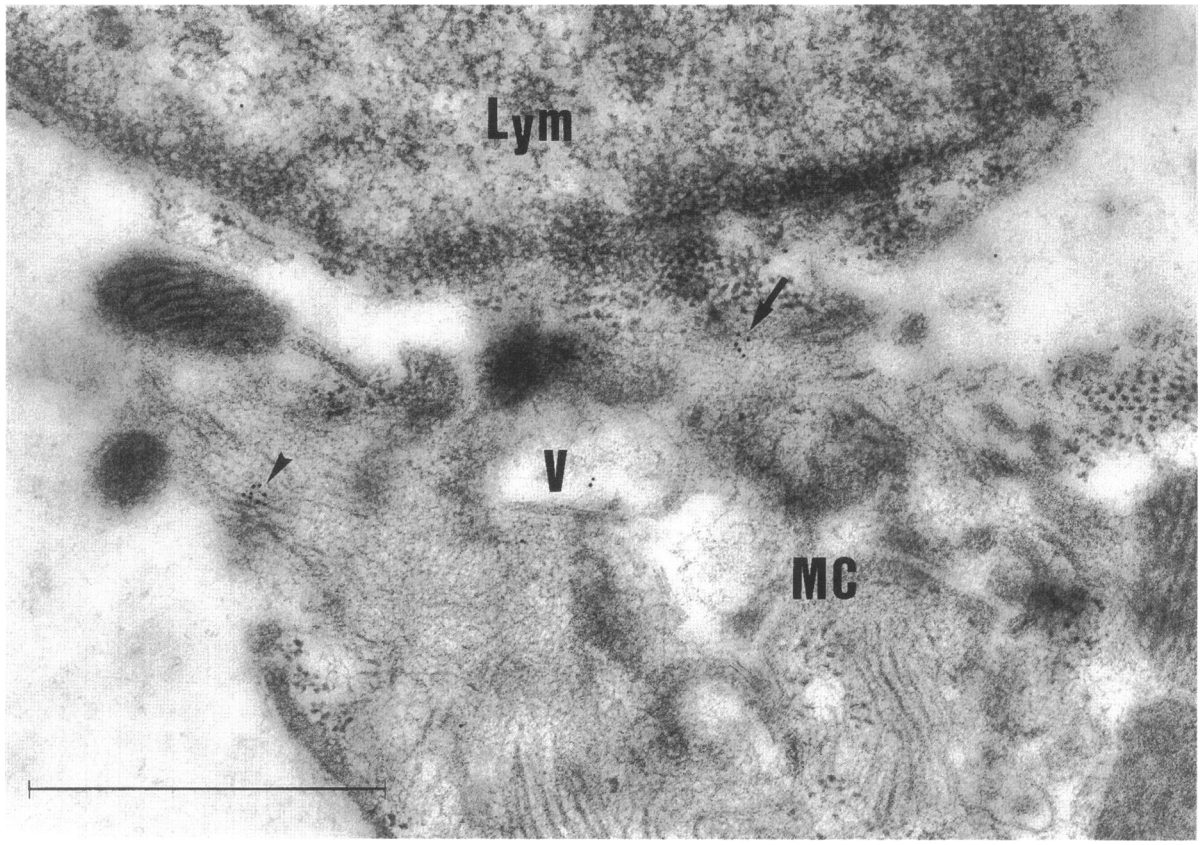

Figure 10. *Immunogold electron micrograph of a cardiocyte (MC) and a lymphocyte (Lym) from a mouse with CVB3 myocarditis on day 8 after virus inoculation. The lymphocyte is in close membrane-to-membrane contact with the degenerated cardiocyte (MC). Hybridization signals are detected in the cytoplasm of the cardiocyte near the point of contact with the lymphocyte* (arrow). *Positive signals are also found in the myofibrils of the cardiocyte near the plasma membrane* (arrowhead). *V, a vesicular structure. Bar, 1 μm.*

Discussion

Group B Coxsackieviruses are considered to be the most common causative agents of viral myocarditis, but the detailed intracellular localization of these viruses in cardiocytes and their infectious cycle have not been clarified. Cell-mediated immune cardiocyte injury is considered to be important in the pathogenesis of Coxsackievirus myocarditis,[6,10,16,28,29,32–38] but a role for direct myocardial damage by the virus was also recently reported.[32]

Light microscopic *in situ* hybridization showed clusters of positive signals for CVB3 RNA in the regions of myocardial necrosis and cell infiltration on days 5 and 8. Signals were detected in the interstitial cells as well as in the cardiocytes, and similar results have previously been reported by several authors.[3–6,10,14–17] These cells were identified by immunohistochemical methods.

We used electron microscopic *in situ* hybridization to examine the location of viral replication and the role of CVB3 RNA in cardiocytes and FL cells.

Poliovirus, one of the enteroviruses, was previously studied in HEP-2 cells by electron microscopic *in situ* hybridization.[21] Troxler et al[21] found hybridization signals abundantly associated with the rER-bound replication complex close to translucent virus-induced vesicles. We found electron-dense products, virus-like particles, and translucent vesicles in the vicinity of the rER in CVB3-infected FL cells, and detected CVB3 RNA in the electron-dense products close to the translucent vesicles. These findings were similar to those obtained in HEP-2 cells infected with poliovirus, suggesting that CVB3 probably replicates in the rER like poliovirus.

In the hearts of mice with CVB3 myocarditis, positive signals were detected in the cytoplasm of apparently intact cardiocytes as well as damaged cells. The signals were detected in the tubular or vesicular structures, near the mitochondria, in the vicinity of the rER, and in the myofibrils. Eisenberg et al[19,20] reported that the density of myosin heavy chain mRNA was approximately 15 times higher in the

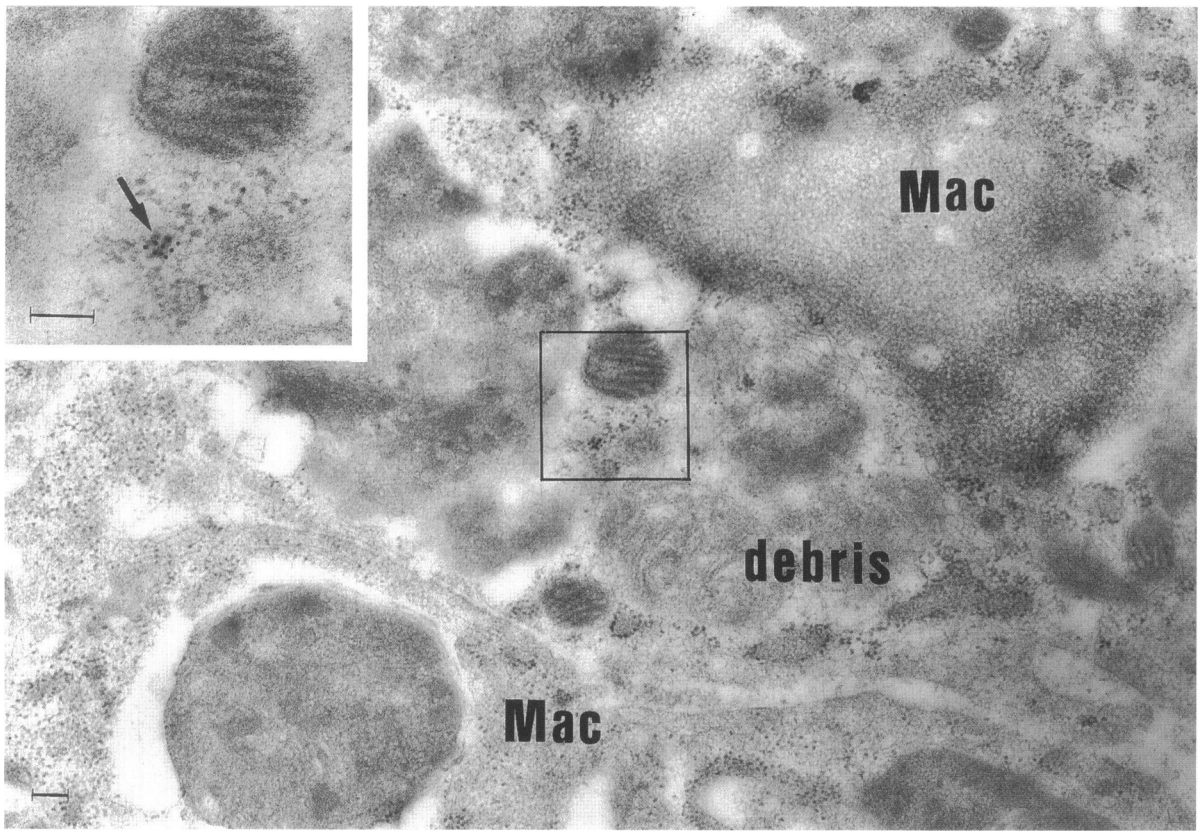

Figure 11. *Immunogold electron micrograph of a necrotic focus from the myocardium of a mouse with CVB3 myocarditis on day 8 after virus inoculation. One of two macrophages (Mac), the upper one has apparently phagocytosed necrotic myocyte debris.* Inset: *Higher magnification of the square inside the macrophage. Positive signals* (arrow) *can be detected in the phagocytosed cell debris. Bars, 0.1 μm.*

cytoskeletal-rich inter-myofibrillar space than in the myofibrils. Detection of CVB3 RNA in the inter-myofibrillar space suggests that the virus may be synthesized on polysomes in this space like myosin heavy chain mRNA. Some of the small vesicular structures with gold particles could not be distinguished from sarcoplasmic reticulum and were sometimes closely associated with the plasma membrane. These findings may indicate that the virus is transferred to the plasma membrane via the vesicular structures after viral RNA is synthesized in the rER or polysomes.

To assess the effects of Coxsackievirus on the myocardium, it is necessary to search for viral antisense RNA and viral proteins.[39] Viral structural proteins have been detected in the myocardium of patients with myocarditis using polyclonal antiserum to a recombinant CVB3 capsid protein, VP1.[40] In the present study, we detected both viral antisense and sense RNA by RT-PCR, not only on days 5 and 8 but also on day 14. Viral RNA was localized to the cytoplasm of cardiocytes and interstitial cells on days 5, 8, and 14 by *in situ* hybridization. The existence of both sense and antisense RNA on day 14, when the virus could no longer be isolated, suggests that the viral RNA was replicated via a double-stranded RNA intermediate[12–16] in a very limited fashion like poliovirus[41] and that RT-PCR or *in situ* hybridization is a more sensitive test than virus isolation.

Cell-mediated immunity has been strongly suggested to have a role in the pathogenesis of acute Coxsackievirus B myocarditis.[10,15,28,29,31–38] Our previous studies have shown that cardiocytes often have membrane-to-membrane contact with cytotoxic T cells in lesions of acute murine CVB3 myocarditis.[28,29] In the present study, infiltrating lymphocytes were often in contact with cardiocytes containing viral RNA. These results suggested that the lymphocytes recognized the major histocompatibility complex antigens of cardiocytes containing viral RNA and possibly caused necrosis by immunomechanisms, but further work will be required to clarify the pathogenesis of CVB3 myocarditis.[10,29,32–38]

In the interstitium, positive signals were found in necrotic cell debris and in the cytoplasm of macrophages. The pseudopod-like cytoplasmic processes of the macrophages were in contact with the necrotic cell debris showing positive signals, and gold parti-

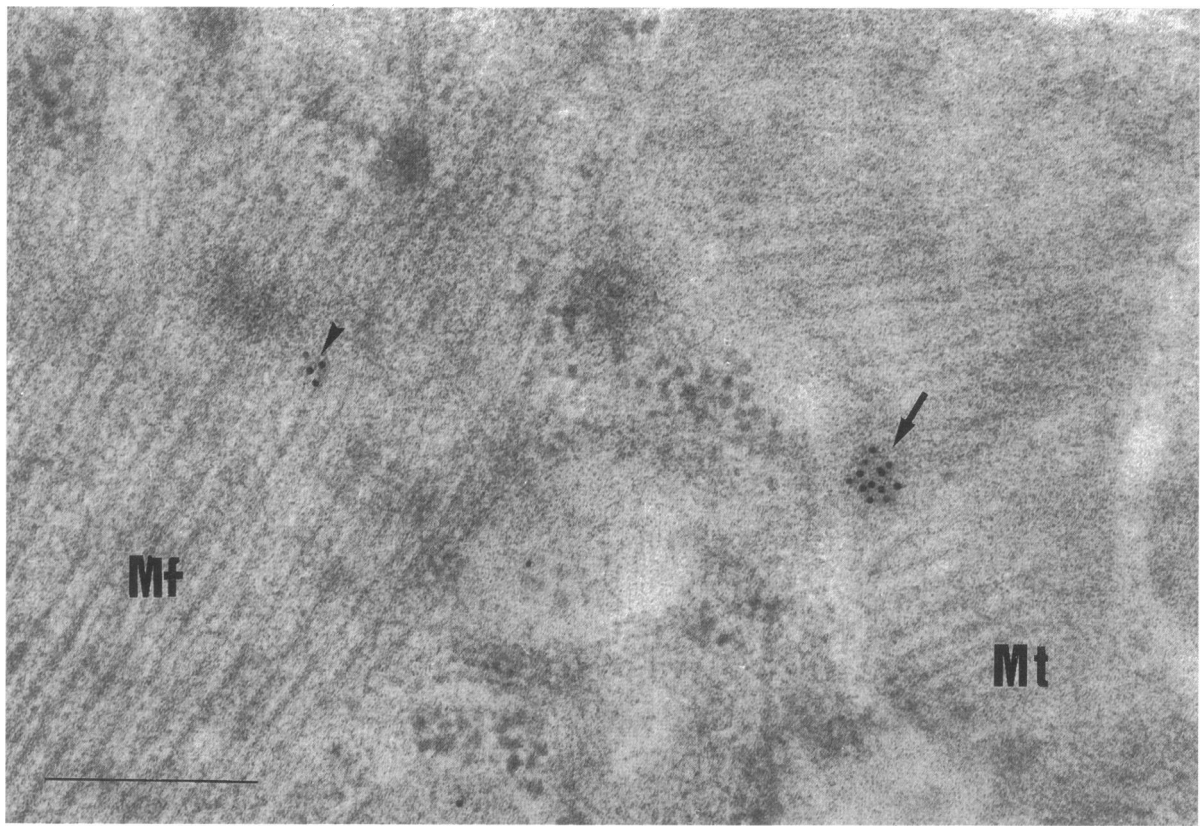

Figure 12. *Immunogold electron micrograph of a necrotic focus from the myocardium of a mouse with CVB3 myocarditis on day 14 after virus inoculation. Gold particles* (**arrowhead**) *are seen on the myofibril (Mf) of a cardiocyte. An aggregate of gold particles* (**arrow**) *is located near the mitochondria (Mt). Bar, 0.1 μm.*

cles were also found in the cytoplasm of macrophages and in their phagocytic vacuoles. Thus, the virus was apparently released from cardiocytes into the interstitium and was then phagocytosed by macrophages.

Signals were still found in the cytoplasm of fibroblasts after the period when the virus could be isolated from the myocardium. Schnurr et al[42] detected persistent Coxsackievirus infection of mouse fibroblasts *in vitro*, and persistent Coxsackievirus infection of YAC-1 cells and human lymphoid cell lines has also been reported.[43,44] The viral genome in fibroblasts may have a significant role in the prolonged positivity of the RT-PCR.

In conclusion, this is the first full report on the intracellular localization of viral RNA by electron microscopic *in situ* hybridization in the myocardium of mice with CVB3 myocarditis. Our results suggest that viral RNA is replicated in the cytoplasm of cardiocytes, transferred to small vesicular and tubular structures, released into the interstitium, and phagocytosed by macrophages. It also appears that lymphocytes recognize virus-positive cardiocytes and that cell-mediated immunity may play an important role in myocardial injury caused by CVB3.

Acknowledgments

We thank Dr. A. Nomoto, Ph.D., Tokyo University, Tokyo, for the kind gift of CVB1 cDNA, and Dr. T Shirai, M.D., Juntendou University, Tokyo, for the kind gift of monoclonal anti-mouse L3T4 antibodies and anti-mouse Lyt2 antibodies. We also thank Mr. M. Shimada and Mr. H. Mukai, from the biotechnology research laboratories of Takara Shuzo Co., for their advice on constructing oligonucleotides.

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