Detection of Viral RNA by Electron Microscopic In situ Hybridization (ISH-EM) in The Germinal Epithelium of Mice Infected with Encephalomyocarditis (EMC) Virus

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Abstract: Electron microscopic in situ hybridization (ISH-EM) was first applied to the detection of viral RNA in the germinal epithelium of mice inoculated i.p. with 10⁶ plaque-forming units/mouse of the D variant of encephalomyocarditis virus (EMC-D). Signals of viral RNA were first detected in a small number of Sertoli cells showing mild degeneration at 2 days post inoculation, and 2 days later, they were also detected in germinal cells and spermatogonia when Sertoli cells showed prominent degeneration. The results clearly demonstrated that the first site of viral attack in the germinal epithelium was Sertoli cell in the case of EMC-D-induced mouse orchitis.

Key words: EMC-D, ISH-EM, orchitis

Encephalomyocarditis (EMC) virus is a cardiovirus which belongs to the family Picornaviridae and it is 20–30 nm in diameter. The D variant of EMC virus (EMC-D) is well known to induce diabetes in particular strains of mice [3] and is now widely used to produce a model of human viral diabetes [15]. Recently we reported first in Syrian hamsters [4, 9] and then in mice [8] that EMC-D is also able to cause orchitis, and EMC-D-induced orchitis especially in mice is now expected as a useful model for human viral orchitis [13].

In our last paper, we investigated the relation between the distribution of viral RNA and the development of histopathological change in the early stage of EMC-D-induced orchitis in mice by light microscopic in situ hybridization (ISH-LM) on paraffin sections. And we got the results suggesting that EMC-D carried to seminiferous tubules via the blood first attacked Sertoli cells and then damaged germinal cells and spermatogonia. However, more clear evidence is needed to confirm that the viral attack occurs later in spermatogonia than in Sertoli cells, because Sertoli cells and spermatogonia have close spacial relationship each other in the basal compartment of germinal epithelium. Therefore, in the present study, we tried to detect viral RNA in the germinal epithelia of EMC-D-infected mice by electron microscopic in situ hybridization (ISH-EM).

Twenty-three 4-week-old male BALB/c mice (Japan SLC, Shizuoka) were used. They were kept under controlled conditions (temperature; 23 ± 2°C, relative humidity; 55 ± 5%) in an isolator caging system (Niki

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shojo Co., Tokyo) and fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo) and water ad libitum. Twenty mice were intraperitoneally inoculated with 10^3 plaque-forming units (PFU)/mouse of EMC-D, and 5 mice each were killed by exanguination under ether anesthesia from 2 to 5 days post inoculation (DPI), respectively. The remaining 3 mice inoculated with 0.01M phosphate buffered saline (PBS) were killed at 5 DPI and served as controls. Small pieces of the testes obtained from each mouse were frozen with dry ice/ethanol and stored at -80°C until used.

Digoxigenin (Dig)-labeled anti-sense RNA probe, which has been prepared in our laboratory [12] and has been shown to be compensatory to the gene encoded specificity region of EMC-D (VP1 region) was used [1, 2]. The specificity of this probe for EMC-D has been confirmed by Northern blotting analysis [12].

ISH-LM was performed according to the Hirasawa’s method [5]. Briefly, cryosections of testes were fixed with 3% paraformaldehyde/PBS for 60 min, 0.2 M HCl for 8 min, 0.25% acetic anhydride/0.1 M triethanolamin-HCl (pH 8.0) for 15 min and 2 x SSC (Saline-sodium citrate buffer) for 10 min. These sections were then prehybridized with 50% deionized formamide/2 x SSC for 60 min. After treatment, the sections were hybridized for 18 hr at 50°C in following solution (probe concentration: 2 μg/ml): 50% deionized formamide, 100 μg/ml yeast t-RNA, 10% dextran sulphate, 1 x Denhardt’s solution, 0.05 M Tris-HCl (pH 7.5), 5 mM EDTA and 0.6 M NaCl. After hybridization, the sections were rinsed with 5 x SSC and washed with 50% formamide/2 x SSC for 30 min at 50°C and incubated with RNase A buffer (2 μg/ml RNase A, 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl) for 30 min at 37°C. The sections were then washed twice in 2 x SSC and 0.2 x SSC (30 min with each) at 37°C.

For colorimetric detection after hybridization, the sections were treated with 1% blocking buffer (Boehringer Mannheim, Tokyo) in DIG buffer I (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl) for 30 min. Sections were incubated with 1:30 anti-Dig antibody directly coupled to ultra-small gold particles (0.8 nm in diameter, Boehringer Mannheim)/blocking buffer for 60 min. The sections were washed in PBS for 10 min and post-fixed with 1% glutaraldehyde/PBS for 5 min at 4°C. The gold particles were enlarged for visualization by silver enhancement (Boehringer Mannheim) for 40 min. Thereafter the sections were dehydrated and embedded in Poly/Bed 812 (Polyscience Co., Ltd., Washington, PA). Semi-thin sections (2 μm) were stained with toluidine blue and subjected to light microscopic examination. On the other hand, ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a JEM-1200EX electron microscope (JEOL Co., Ltd., Tokyo).

Findings of ISH in the present study were similar to those of ISH-LM on paraffin section in our previous report [13]. Namely, signals of viral RNA were first detected in a few Sertoli cells in almost intact germinal epithelia at 2 DPI, and the number of Sertoli cells bearing signals of viral RNA increased at 3 DPI fight when mild degenerative changes were rarely found in germinal epithelia. At 4 DPI, signals of viral RNA came to be detected not only in Sertoli cells but also in a small number of germinal cells and spermatagonia.

In the examination by ISH-EM, signals of viral RNA were first appeared in the cytoplasm of a small number of Sertoli cells showing mild degeneration at 2 DPI, and the number of Sertoli cells bearing signals of viral RNA increased at 3 DPI (Fig. 1). At that time, however, germinal cells as well as spermatagonia adjacent to Sertoli cells bearing viral signals were completely free from signals of viral RNA (Figs. 2 and 3). In germinal cells and spermatagonia, signals of viral RNA appeared first at 4 DPI when Sertoli cells showed promin...
nent degeneration. These findings of ISH-EM confirmed those of ISH-LM in our present and previous studies and directly demonstrated that EMC-D attacks first Sertoli cells in the germinal epithelium.

Although why the virus attack occurred later in spermatogonia than in Sertoli cells is still unclear, there may be a difference in the expression of receptors for EMC virus between Sertoli cells and spermatogonia. In this regard, Huber [6] reported that VCAM-1 acted as a receptor for EMC-D, and Riccioli et al. [7] demonstrated that VCAM-1 was expressed on the cell surface of Sertoli cells in the germinal epithelium. We are now examining the mode of expression of VCAM-1 on Sertoli cell's surface during EMC-D-infection.

ISH-EM has been applied to the studies on ultrastructural localization of nuclear matrices using pre-embedding method in combination with ultra-small gold antibody and silver enhancement treatment up to the present time [10, 11, 14]. However, this is the first report that ISH-EM has been applied to the detection of viral RNA. ISH-EM seems to be very useful for the detection of intracellular localization of a small signals of viral nucleic acid especially in the early stage of viral infection.

Fig. 2. Basal compartment of germinal epithelium of an EMC-D-infected mouse at 3 DPI. Gold particles are seen in the cytoplasm of a degenerated Sertoli cell (S) but not in a spermatogonium (G). SH-EM, Bar=2 μm.

Fig. 3. Basal compartment of germinal epithelium of an EMC-D-infected mouse at 3 DPI. Gold particles are seen in the cytoplasm of a degenerated Sertoli cell (S) but not in a spermatogonium (G) and germinal cells. ISH-EM, Bar=1 μm.

References