Detection of Canine Herpesvirus DNA in the Ganglionic Neurons and the Lymph Node Lymphocytes of Latently Infected Dogs

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ABSTRACT. To determine the site of latent infection of canine herpesvirus (CHV), tissues from dogs convalescent from acute infection with CHV were examined for the presence of viral genome DNA by the nested polymerase chain reaction. CHV DNA was detected in the trigeminal ganglia and the retropharyngeal lymph nodes. *In situ* hybridization study of the tissues revealed that CHV genome persisted in the nuclei of ganglionic neurons and lymphocytes.—**KEY WORDS:** canine herpesvirus, ganglionic neuron, latency, lymphocyte.

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Canine herpesvirus (CHV) causes a generalized necrotizing and hemorrhagic disease in neonatal pups, and usually subclinical upper respiratory infection in adult dogs [1]. It has been indicated that CHV persists in the trigeminal ganglion or a variety of tissues of naturally infected dogs as viral DNA, and that stress or administration of corticosteroid leads reactivation of the virus [4, 14]. CHV belongs to the alphaherpesvirus group, containing feline herpesvirus 1 (FHV-1), equine herpesvirus 1 (EHV-1), bovine herpesvirus 1 (BHV-1) and pseudorabies virus (PRV) [18]. Recent studies demonstrated that the genomes of alphaherpesviruses harbored in the ganglionic neurons [2, 13, 17, 19] as well as in lymphocytes during latent infection [5].

To determine cell types where CHV latently infects, tissues of dogs convalescent from experimental infection with the virus via different routes were examined for the viral DNA by using nested polymerase chain reaction (PCR) and *in situ* hybridization.

MATERIALS AND METHODS

Virus, cells and animals: The GCH-1 strain of CHV was used for all inoculations. The origin and history of the strain were presented in previous report [8]. The strain was passaged 40 times and propagated in Madine-Darby canine kidney (MDCK) cells. The cells were grown in modified Eagle's minimal essential medium (MEM) supplemented with 5% calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and nonessential amino acids. Nine adult (>2 years) dogs of female weighing 10.8–14.7 kg were used in this study. Nos. 1 and 2 were mongrels, others were beagles. They were all seronegative to CHV at the time of inoculations. The viruses of 2×10^6 TCID₅₀ were inoculated intranasally to two dogs (Nos. 1 and 2). The same doses of the virus were inoculated both intravenously and intranasally to three animals (Nos. 3, 4 and 5). The same doses of the virus were inoculated intravaginally to three animals (Nos.

6, 7 and 8).

Virus isolation and titration: Nasal and vaginal swabs were taken every 2nd day for 2 weeks after inoculation with CHV. Thereafter, the swabs were collected every other week until dogs were dissected. All swabs were placed in chilled MEM and kept in -80°C until use. After thawing the samples, serial 10-fold dilutions were prepared in MEM and 50 µl amount of each dilution was inoculated onto monolayers of MDCK cells in 96-well tissue culture plates.

Collection of materials: Two or four months after inoculation, the animals were sacrificed and tissues listed in Table 2 were collected. Peripheral blood mononuclear cells (PBMCs) were collected from 2 ml of blood by using Ficoll-Paque reagent (Pharmacia Biotech). These collected samples were kept at -40°C or fixed with para-formaldehyde for immunological staining and for *in situ* hybridization.

DNA extraction from the tissues and nested PCR: Genomic DNA was extracted from tissue homogenates (10 mg) digested by proteinase K, using phenol and chloroform [20]. CHV immediate early (IE) gene within the inverted repeats was amplified by nested PCR using the genomic DNA (Fig. 1) [3]. The first PCR was performed in a 25 µl volume containing 0.25 µl of DNA, 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTP mixture, 1.25U Taq DNA polymerase (GIBCO BRL), 50 µM IE-1 primer (5'-GATAATTCAGCTTCTAGCGATG-3') and 50 µM IE-2 primer (5'-GATCTCACATC TATAGTTTGGAG-3'). After denaturation at 94°C for 1 min, amplification was carried out for 30 cycles: 45 sec of denaturation at 93°C, 30 sec of annealing at 56°C, and 1 min of extension at 71°C. The final cycle was terminated with a 3 min extension at 71°C. The PCR products were diluted in distilled water to give 1/10 concentrations. The nested PCR was performed in a 25 µl volume containing 1 µl diluted PCR products, 50 µM IE-1N primer (5'-AACCAACTCCAGCTAAAGCAT-3'), 50 µM IE-2N

Fig. 1. Schema of the CHV genome and the target of nested PCR. (A) Schema of the CHV genome. UL:unique long region. IRs:internal repeats. US:unique short region. TRs:terminal repeats. (B) The *Hind* III d and g fragments. The CHV IE ORF is shown by arrow. (C) The summary of primers used in nested PCR. Direction are shown by arrows. The length of the DNA fragment produced by nested PCR is 355 bps.

primer (5'-GTTGATTCATTAGGTAAAGCATT-3') as above. The primer pairs of IE-1/IE-2 and IE-1N/IE-2N were synthesized according to the sequences reported previously [10].

Virus isolation from the tissue homogenates: Nerve ganglia and lymph nodes were homogenized in MEM to give concentrations of 10%, then inoculated to MDCK cells and 3 passages were made at weekly intervals.

Immunohistochemical staining: The streptavidin biotin complex (SAB) method was applied to the trigeminal ganglia and retropharyngeal lymph node sections to detect viral antigens. Rabbit immune serum against CHV virions was used as primary antibodies. For antibody detection the Histofine SAB-Kit was used according to the manufacturer's instruction (Nichirei).

In situ hybridization: Tissues were fixed in 4% paraformaldehyde for 3 or 4 days at 4°C and embedded in paraffin for sectioning. The tissue sections on glass slides were washed in xylene for 20 min to remove paraffin. The sections were dehydrated in graded ethanol solutions and washed twice in 0.1 M phosphate buffer (pH 7.4, PB) for 15 sec, treated with proteinase K (1–200 µg/ml) for 15 min at 37°C, and fixed in 4% paraformaldehyde for 10 min. As a control, the proteinase K-digested sections were treated with RNase-free DNase (200 µg/ml) for 1 hr at 37°C. Then the sections were washed in PB for 1 min and treated with 0.2 N HCl for 10 min, washed in PB for 1 min, dipped in 0.1 M triethanolamine-0.25% (vol./vol.) acetic anhydride for 10 min, washed in PB for 1 min, and dehydrated in 80% and 100% ethanol for 3 min. The slides were dried completely on hot plate at 50°C for 2 hr. Sections were hybridized with 25 ng of the CHV IE DNA probe labeled with Digoxigenin (DIG)-TTP by PCR using primers IE-1N and IE-2N, per slide for 16 hr at 42°C in 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 45% formamide, 10% dextran sulphate, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 × Denhart's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02%

Ficoll) and 1.0 mg/ml tRNA of brewer's yeast. Following hybridization, slides were washed in 5 × SSC for 1 min, 2 × SSC and 50% formamide for 30 min, and washed twice in 0.2 × SSC for 20 min each at 42°C. Detection of hybrids with anti-DIG alkaline phosphatase-conjugate were performed by use of X-phosphate and nitro blue tetrazolium according to the manufacture's instructions (Boehringer Mannheim).

RESULTS

Virus recovery from the dogs inoculated with CHV via different routes: CHV was isolated only from the nasal swabs of dogs inoculated intranasally or both intranasally and intravenously until four or six days after inoculation. On the other hand, CHV was isolated from both nasal and vaginal swabs of dogs inoculated intravaginally until four or six days after inoculation (Table 1). No dog showed clinical signs. Serum neutralization antibody titers of all animals increased until three or four weeks after inoculation, then decreased (data not shown).

Detection of CHV genome DNA in the tissues of convalescent dogs by nested PCR: The results of nested PCR of DNA templates extracted from various tissues of nine dogs are shown in Table 2. All animals harbored CHV genome in the trigeminal ganglia, irrespective of inoculation routes. The viral DNA was detected in the retropharyngeal lymph nodes of 7 of 8 dogs. CHV genome DNA was also detected in the lumbosacral ganglions (Nos. 2, 3, 6 and 7), tonsils (Nos. 2 and 3), mediastinal lymph nodes (No. 3), thymus (No. 3) and hypogastic lymph nodes (Nos. 6, 7 and 8). The CHV DNA was not detected in the PBMCs by nested PCR. The amplified fragment was confirmed as a portion of CHV genome by Southern blot hybridization (data not shown). The sensitivity of nested PCR/Southern blot analysis were equivalent to 1 TCID₅₀. No virus was recovered from the tissue homogenates and no antigen was detected on these tissue sections by immunohistochemical staining using rabbit hyper-immune serum against CHV virions. Treatment of the convalescent animals with dexamethasone increased serum neutralization antibody titers, although no virus was recovered (data not shown). These findings indicate latent infection in the animals by CHV.

In situ hybridization of CHV genome in the trigeminal ganglia and the retropharyngeal lymph nodes: In order to identify the cell types where CHV genome harbored during the phase of latent infection, *in situ* hybridization was performed on sections of the trigeminal ganglia and the retropharyngeal lymph nodes of animals latently infected with CHV. Of the 6 ganglia tested, 4 (Nos. 1, 4, 6 and 7) were positive for CHV genome. Signals were found only in the nuclei of the neurons (Fig. 2). Positive rate of neurons latently infected with the virus were between 0.01% to 0.04%. No signal was observed in the control sections treated with DNase and in the sections hybridized with DIG-labeled probe solution containing excess amount of

Table 1. Isolation of CHV from swabs after inoculation

Animal No.	Swabbed sites	Days after inoculation					
		2	4	6	8–14 ^{b)}	28–56	70–112
Intranasal inoculation							
1	Nose	4.0 ^{a)}	3.5	–	–	–	
	Vagina	–	–	–	–	–	
2	Nose	3.67	4.0	1.0	–	–	–
	Vagina	–	–	–	–	–	–
Intranasal and intravenous inoculation							
3	Nose	4.33	3.5	0.67	–	–	–
	Vagina	–	–	–	–	–	–
4	Nose	4.0	3.33	0.5	–	–	–
	Vagina	–	–	–	–	–	–
5	Nose	4.33	2.0	–	–	–	–
	Vagina	–	–	–	–	–	–
Intravaginal inoculation							
6	Nose	–	1.0	–	–	–	
	Vagina	2.5	2.0	–	–	–	
7	Nose	–	0.83	–	–	–	
	Vagina	1.0	2.33	1.5	–	–	
8	Nose	–	3.67	0.67	–	–	
	Vagina	2.0	3.5	–	–	–	
Control (not inoculated)							
9	Nose	–	–	–	–	–	–
	Vagina	–	–	–	–	–	–

a) Virus titers are expressed as log₁₀TCID₅₀/50 µl.

–: No virus was isolated.

b) 8–14: Samples were collected every 2nd days.

28–56: Samples were collected two weeks apart.

56–112: Samples were collected two weeks apart.

Table 2. Detection of CHV genome in the tissues of latently infected dogs

Tissue	Animal No.								
	1 ^{a)}	2	3	4	5	6	7	8	9
Cerebrum	– ^{b)}	–	–	–	–	–	–	–	–
Cerebellum	–	–	–	–	–	–	–	–	–
Cornea	–	–	–	–	–	–	–	–	–
Lacrimal gland	–	–	–	–	–	–	–	–	–
Nasal swab	–	–	–	–	–	–	–	–	–
Parotid gland	–	–	–	–	–	–	–	–	–
Trigeminal ganglion	+	+	+	+	+	+	+	+	–
Tonsil	–	+	+	–	–	–	–	–	–
Retropharyngeal lymph node	+	+	+	+	+	–	+	+	–
Axillares lymph node	nc	–	–	–	–	–	–	–	–
Thymus	–	–	+	–	–	–	–	–	–
Lung	–	–	–	–	–	–	–	–	–
Pulmonales lymph node	–	–	+	–	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–	–
Spleen	–	–	–	–	–	–	–	–	–
Kidney	–	–	–	–	–	–	–	–	–
Lumbosacral ganglion	–	+	+	–	–	+	+	–	–
Hypogastric lymph node	–	–	–	–	–	+	+	+	–
Mammary lymph node	nc	–	–	–	–	–	–	–	–
Endometrium	–	–	–	–	–	–	–	–	–
Vaginal swab	–	–	–	–	–	–	–	–	–
PBMCs	–	–	–	–	–	nc	nc	nc	–

a) Nos.1 and 2 :intranasal inoculation. Nos.3, 4 and 5 :intranasal and intravenous inoculation. Nos. 6, 7 and 8 :intravaginal inoculation. No.9 :control (not inoculated).

b) +: Positive. -: Negative. nc: not collected.

Fig. 2. *In situ* hybridization on the trigeminal ganglia and retropharyngeal lymph nodes from latently infected dogs. Positive signal was detected in the nucleus of a neuron in the trigeminal ganglion (a) and the nucleus of a lymphocyte in the retropharyngeal lymph node (c) ($\times 200$). DNase-treated section of the trigeminal ganglion (b) and retropharyngeal lymph node (d) ($\times 200$).

unlabeled CHV DNA. In the retropharyngeal lymph nodes, CHV genomes were detected in the nuclei of lymphocytes from 3 animals (Nos. 2, 4 and 8) (Fig. 2). Frequency of positive lymphocytes ranged from 1×10^{-9} to 5×10^{-7} .

DISCUSSION

In the present study, CHV genome was detected in the trigeminal ganglia of dogs experimentally infected with CHV, while infectious virus was not recovered from the tissue homogenates. These findings indicate that CHV genome remained latent in the animals. During the acute phase, all dogs shed the virus in the nasal secretions irrespective of routes of inoculations, showing that CHV replicated in their nasal mucosa. Although it was uncertain whether the animals were "reinfected" with the virus shed in the vaginal secretions or systemic infection occurred, the virus must be transported to the trigeminal ganglia from sensory nerve endings [6, 16]. *In situ* hybridization study demonstrated that CHV genome harbored in the nuclei of neurons in the trigeminal ganglia. The trigeminal ganglia of animals have been identified as the site of latent infection of FHV-1, EHV-1, BHV-1 and PRV [2, 7, 13, 17].

CHV genome was also detected in the lumbosacral ganglia of two animals inoculated with the virus intranasally or both intranasally and intravenously in the present study. Although no virus was recovered from vaginal swabs of these animals, CHV must replicate in the epithelial cells of vagina and be transported to the ganglia. It was suggested that the lumbosacral ganglion was an important site for latency and that recrudescence virus played an important role in venereal infections [4]. Since CHV infection in pregnant dogs causes abortion and stillbirth, the mechanism of reactivation of CHV should be clarified [9, 15].

CHV genome was also detected in the retropharyngeal lymph nodes of all but one animal, and detected in the hypogastric lymph nodes of those inoculated intravaginally. These results suggest that the lymphocytes were infected

with the virus that replicated in epithelial cells of mucosa and migrated to these lymph nodes. In the animals intravenously inoculated with CHV, viral genome was detected in a variety of lymphoid tissues. This finding suggests that CHV genome circulate systemically via blood or lymphatic fluid as shown with other herpesviruses [5, 11, 12]. In the present study, CHV genomic DNA was not detected in PBMCs. To make clear whether the infected lymphocytes circulate systemically, further investigations are necessary. It was reported that PRV-infected blood mononuclear cells caused abortion by transmitting the virus to placental cells [12].

Latent infection of herpesviruses is implicated in a variety of diseases following reactivation [21]. The present results provided information of CHV latency, and the usefulness of PCR and *in situ* hybridization techniques for the diagnosis of latent CHV infection.

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