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Detection of HSV mRNA Using Reverse Transcription-polymerase Chain Reaction for Diagnosis in Murine Herpetic Keratitis Model

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Abstract: Reverse transcription polymerase chain reaction (RT-PCR) was applied in the detection of herpes simplex virus-1 (HSV-1) mRNA from tear film and corneal epithelium in a murine herpetic keratitis model. The diagnostic value of this new technique for acute herpetic keratitis was evaluated in comparison with direct PCR for genomic DNA and viral culture. On day 2 postinfection (PI) of HSV, all mice showed dendritic keratitis, and PCR, RT-PCR, and viral culture were positive in all samples. On day 8 PI, no dendritic keratitis was observed in any mouse, PCR was positive in all samples, while RT-PCR was positive in only 5 of 12 samples and viral culture in only 2 of 12. The sensitivity of RT-PCR was lower than that of PCR, and approximately the same as viral culture; however, the findings of RT-PCR more closely concurred with clinical observations than the findings of PCR. These results show the potential of RT-PCR for rapid, specific diagnosis of acute herpetic keratitis. **Jpn J Ophthalmol 1998;42:8–11** © 1998 Japanese Ophthalmological Society

Key Words: Acute herpetic keratitis, diagnosis, reverse transcription polymerase chain reaction.

Introduction

Herpetic keratitis is one of the major causes of corneal blindness; therefore, the rapid and accurate detection of herpes simplex virus (HSV) is important, particularly in atypical cases.

Recently, polymerase chain reaction (PCR) has been used in medical virology to detect viral nucleic acid.[1,3,6] In ophthalmology, PCR has been applied constructively in the DNA diagnosis of herpetic infection. However, since HSV establishes latent host infection characterized by the presence of viral DNA but restricted viral transcripts,[12] PCR may show a positive result with latent virus that may remain inactive in ocular tissues. Indeed, there are several reports of HSV DNA detection by PCR from tear films in quiescent herpetic keratitis,[15] or from corneal buttons with[4,9,10,13] and without past history.[4,9] Accordingly, the high sensitivity of the PCR technique makes it unsuitable for the diagnosis of active HSV infection.

The detection of mRNA is considered theoretically to be more appropriate for diagnostic use than the detection of DNA, since the presence of mRNA usually accompanies viral replication.[11] Reverse transcription (RT)-PCR has recently been used to detect the mRNA of cytomegalovirus from peripheral blood leukocytes.[2] Using this method, mRNA is converted to cDNA by reverse transcriptase, providing cDNA for PCR. In practice, the detection of mRNA from tear film and corneal epithelium has been thought to be quite difficult because of the presence of RNase in tear film and the instability of mRNA. To our knowledge, this is the first report of the detection of HSV mRNA from tear film and corneal epithelium in herpetic keratitis.

In this study, using RT-PCR, we succeeded in detecting HSV-1 mRNA in small volumes of samples. We studied the diagnostic value of RT-PCR for murine HSV-1 keratitis, in comparison with viral cul-

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ture and direct PCR detection of genomic DNA from tear film and corneal epithelium.

Materials and Methods

Virus and Animals

The Kawamura[7] strain of HSV-1 was used for inoculation. A stock of the virus was grown in vero cells. All mice were 6-week-old female BALB/c. After induction of anesthesia, right corneas were scarified with a 27-gauge needle; a 10 μL virus suspension containing 10^4 pfu of HSV-1 was placed on the corneal surfaces, and the eyes were rubbed. Animals used as controls were inoculated with saline by the same technique. The lesions were observed daily.

Preparation of Samples

Tear film and corneal epithelium samples were collected from each eye with two swabs at 2, 4, and 8 days post-infection (PI). The swab for PCR assay was immediately placed in a microfuge tube containing 50 μL PCR buffer (10 mmol/L Tris-hydrochloride, pH 8.3), 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride (Toyobo, Osaka), 100 μg/mL fresh proteinase K, 0.5% Tween 20[8] and RNase inhibitor (Takara Shuzo, Osaka).

The other swab was placed on confluent vero cell culture for virus isolation and assayed as previously described.[5] All culture specimens were monitored for HSV-induced cytopathic effect for 2 weeks.

Nucleic Acid Preparation

The microfuge tubes with PCR samples were incubated for 60 minutes at 55°C to digest protein; 10 minutes at 95°C to inactivate the proteinase, and then quenched on ice. RNase inhibitor was then added, followed by storage at −80°C to preserve RNA.[8]

PCR Primers

The primers used for detecting HSV-1 DNA or mRNA were chosen from the region of the HSV-1 DNA polymerase gene (Genemed, USA) which amplified a fragment of 106 base pairs.

PCR Amplification

The PCR protocol described by Saiki[14] was used, with some modifications. Ten-μL aliquots of nucleotide acids were used for each reaction. PCR was performed in a 100 μL reaction volume containing the PCR buffer with 0.2 mmol/L of each dNTP, 50 pmol/L of each 5′ and 3′ primer, and 2.5 units of Tth DNA polymerase (Toyobo). The reaction mixture was overlaid with mineral oil. Reactions were followed by 32 cycles, consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. Aliquots from each reaction mixture were fractionated electrophoretically on 2% agarose gel containing ethidium bromide; PCR products were then visualized under ultraviolet light.

RNA Preparation and cDNA Synthesis

Prior to cDNA synthesis, each nucleic acid sample was digested with RNase-free DNase (Takara, Osaka) at 37°C for 60 minutes, then 10 minutes at 95°C. Ten μL aliquots of RNA were used for each reaction. The cDNA was generated as follows: RNA was incubated at 45°C for 1 hour in a total volume of 20 μL PCR buffer with 100 pmol/L of random hexamer primers (Takara), 0.2 mmol/L of each dNTP, 20 U of avian myeloblastosis virus reverse transcriptase (Takara) and RNase inhibitor. After reverse transcription of RNA into cDNA, the resulting cDNA/RNA heteroduplex was denatured at 95°C for 5 minutes. PCR was performed in a 100 μL reaction volume by adding 80 μL of the PCR buffer, 50 pmol/L of each 5′ and 3′ primer, and 2.5 units of Tth DNA polymerase, as described for direct PCR. All procedures including cDNA synthesis and PCR, were performed in identical microfuge tubes.

Each DNase-treated sample without reverse transcription (RT(-)-PCR) was also subjected to PCR to confirm the absence of contaminating residual genomic DNA.

Identification of PCR Product

Southern blot hybridization was carried out to identify the specificity of PCR products. In brief, after electrophoresis of the PCR amplification in agarose gel, DNA samples were transferred to a nylon filter and fixed by UV cross linker. The filter was prehybridized at 68°C for 60 minutes in hybridization solution [5×SSC(1×SSc = 150 mmol/L sodium chloride, 15 mmol/L sodium citrate], 1% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.1% N-lauroylsarkosine, 0.02% sodium dodecyl sulfate (SDS)]. The filter was then hybridized at 55°C for 12 hours with an oligonucleotide probe specific for HSV-1 PCR products labeled with digoxigenin (DIG) (Genemed). For probe labeling, DIG oligonucleotide tailing kit (Boehringer, Mannheim) was used. After membrane washing with 2×SSC, 0.1% SDS at 55°C for 5 minutes (twice) and 0.1×SSC,

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0.1% SDS at 55°C for 5 minutes (twice), immunological detection for color developing was carried out using DIG nucleic acid detection kit (Boehringer instructions). Labeling and detection were performed according to kit inserts.

Results

RT-PCR for HSV mRNA

We could detect HSV mRNA in tear film and corneal epithelium samples by means of RT-PCR. The primers made it possible to detect RNA transcripts from the DNA polymerase region of HSV-1 (Figure 1). The primers designed for this gene amplified the same size of fragment (106 bp) from genomic DNA and cDNA. In two samples, both RT-PCR and RT(-)-PCR showed positive results; therefore, these two bands were not identified as of cDNA origin (Table 1). However, positive results with RT-PCR and negative results with RT(-)-PCR were obtained in other samples, so the origins of these other bands could be confirmed.

Murine Herpetic Keratitis Model

As shown in Table 1, on day 2 PI, all mice showed dendritic keratitis; PCR, RT-PCR, and viral culture were positive in all sample. On day 4 PI, dendritic keratitis was observed in 9 of 12 mice; both PCR and viral culture were positive in all samples, while RT-PCR was negative in two samples. On day 8 PI, no dendritic keratitis was observed in any mouse. Blepharitis was observed in 2 of 12 mice. Stromal lesions in these mice began to develop around day 6, and reached peak severity between days 10 to 14. PCR was positive in all samples, while RT-PCR was positive in only 5 of 12, and viral culture in only the two samples taken from the cases showing blepharitis.

The mRNAs were almost cleared on day 8 PI, corresponding to the disappearance of clinical symptoms; however, DNAs remained positive for some time after clinical symptoms disappeared.

All positive samples were confirmed by Southern blot hybridization with HSV probe. As described above, RT(-)-PCR was positive in two samples on day 4 PI; therefore the origins of these samples were not identified. Samples obtained from negative controls did not show positive results in any assays.

Discussion

The detection of viral mRNA facilitates discrimination between latent and active infection, since the former should lack the synthesis of structural proteins. Latent HSV infection is characterized by extremely restricted viral transcription; its only gene transcripts to have been detected in latency being the latency-associated transcripts (LATs). In one study, viral RNA sequences equivalent to LATs were detected in human trigeminal ganglia by in situ hybridization.[16] Another investigation demonstrated that LAT sequences could be detected by RT-PCR from corneal buttons with quiescent herpetic keratitis.[9] There has been no evidence of viral transcripts other than LATs during latency.

As we describe in this report, RT-PCR can detect HSV mRNA from very small amounts of tear film and corneal epithelium in a murine herpetic keratitis model. The primers used were encoding for DNA polymerase classified as β protein, which is not

Table 1. Detection of HSV-1 in Tear Film and Corneal Epithelium by Viral Culture, DNA-PCR, and RT-PCR

	2 days postinfection				4 days postinfection				8 days postinfection			
Samples	Viral culture	DNA-PCR	RT-PCR	Lesion	Viral culture	DNA-PCR	RT-PCR	Lesion	Viral culture	DNA-PCR	RT-PCR	Lesion
1	+	+	+	dendrite	+	+	+	healed	−	+	+	healed
2	+	+	+	dendrite	+	+	+	dendrite	−	+	+	healed
3	+	+	+	dendrite	+	+	N.I.	healed	−	+	−	healed
4	+	+	+	dendrite	+	+	N.I.	dendrite	−	+	−	healed
5	+	+	+	dendrite	+	+	+	dendrite	−	+	−	healed
6	+	+	+	dendrite	+	+	+	dendrite	+	+	+	blepharitis
7	+	+	+	dendrite	+	+	+	dendrite	−	+	+	punctate
8	+	+	+	dendrite	+	+	−	dendrite	−	+	−	healed
9	+	+	+	dendrite	+	+	−	dendrite	−	+	−	healed
10	+	+	+	dendrite	+	+	+	healed	−	+	−	healed
11	+	+	+	dendrite	+	+	+	dendrite	−	+	−	healed
12	+	+	+	dendrite	+	+	+	dendrite	+	+	+	blepharitis

N.I.; not identified

found during latency; the presence of this transcript therefore indicates viral replication.

Although viral culture is considered to be the most specific method of diagnosing viral infection, its applicability in clinical diagnosis appears to be limited, because it takes approximately 3 to 14 days to yield positive results. On the other hand, although PCR appears to be rapid and more sensitive in detecting HSV DNA than other techniques, its diagnostic value has been controversial because of low specificity.

The results of the present study show that PCR sensitivity for DNA was higher than the sensitivity of both viral culture and RT-PCR in specimens taken at 8 days PI. In other words, PCR was positive in all specimens even at 8 days PI, when all the lesions had healed. The presence of DNA in these specimens could be explained by the presence of DNA in persistent infection or residual DNA fragments of viral particles, RT-PCR, by contrast, was positive in only 5 of 12, and viral culture in only 2 of 12. The findings of the latter two procedures agreed more with clinical observation than the findings of direct PCR.

The results of our study suggest that RT-PCR can be considered as sensitive for mRNA detection as viral isolation and more specific for diagnosing acute herpetic infection than PCR, which is useful in detecting genomic DNA. As demonstrated, RT-PCR is rapid, giving results on agarose gel within 1 day and hybridization results within 3 days. Accordingly, RT-PCR appears to be a useful tool in the diagnosis of acute herpetic keratitis.

Further study is now in progress to evaluate the use of RT-PCR human clinical samples.

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