***In situ* Hybridization for the Detection of Feline Interleukin 1 α mRNA on the Paraffin-Embedded Section using Biotin-Labeled Probes**

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ABSTRACT. *In situ* hybridization (ISH) technique with a biotin-labeled probe was established for detecting feline interleukin 1 (IL-1) α mRNA in necropsied specimens. Homology between human IL-1 α cDNA used as a probe and feline IL-1 α mRNA was confirmed by means of dot blot hybridization using the biotin-labeled probe. Hence, we tried by this biotinylated probe to detect mRNA of IL-1 α in paraffin-embedded sections. The following results were obtained for the routine procedures: 1) coating slides with poly-L-lysine and/or heating at 60°C at least for 6 hours gave an excellent result for the adhesion of the tissue sections, 2) 10 μ g/ml solution of proteinase K treatment for 30 minutes or 50 to 100 μ g/ml solution of proteinase K treatment for 10 to 30 minutes at 37°C gave the good results in the detection of ISH signal, 3) suitable denaturation time of probes at 70 to 90°C was 5 to 15 minutes, and 4) effective hybridization was obtained by incubation for 24 hours at 4°C, for 18 to 24 hours at 25°C or for 5 to 24 hours at 37°C—KEY WORDS: feline, IL-1, *in situ* hybridization, mRNA, paraffin-embedded section.

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In situ hybridization (ISH) is a useful technique for the detection of specific nucleic acid sequences in tissues and cultured cells, and provides a lot of new biological informations [2, 4, 7, 12–14, 16–19]. Recently, many attentions have been focused on the usefulness of ISH analysis using non-radioactive probe, because this technique has the potential for broad application in the pathology. However, ISH analysis of paraffin-embedded tissues using non-radioactive probe, especially biotin-labeled probe, have been limited [2, 12, 13, 16, 19]. In the present study, we attempted to apply the ISH procedure with a biotinylated probe for interleukin 1 α (IL-1 α) to paraffin-embedded sections from a cat with feline infectious peritonitis (FIP) in which the overproduction of IL-1 had been reported [11].

MATERIALS AND METHODS

Feline peritoneal macrophages: Peritoneal macrophages (PM) from healthy cats were elicited by intraperitoneal injection of 50 ml of thioglycollate broth (Difco Laboratories, Detroit, MI, U.S.A.). PM were harvested by sterily washing the peritoneum with phosphate buffered saline (PBS, pH 7.4) 4 days after injection of thioglycollate broth. PM were washed 3 times and then incubated in RPMI 1640 medium supplemented with 10% fetal calf serum and L-glutamine. The resultant cell

preparations contained about 85% macrophages.

RNA preparation: After removing nonadherent cells, PM were stimulated for 6 hr with 1 μ g/ml of lipopolysaccharide (LPS) (Sigma, St Louis, MO, U.S.A.) in tissue culture medium [9, 11, 18], chilled on ice, scraped, and transferred to a sterile tube. These cells were centrifuged at 1,000 rpm for 7 min, and the precipitated cells were suspended in 2 ml of medium. LPS-treated and non-treated PM were treated with 5 ml of NP40 lysis buffer containing 1.4 mM NaCl, 0.015 mM MgCl₂, 0.1 mM Tris (pH 8.6) and 5% ribonucleoside vanadyl complex (Sigma, St Louis, MO, U.S.A.), incubated for 10 min at 4°C, added with 125 μ l of 20% Nonidet P40 (Sigma, St Louis, MO, U.S.A.), and then centrifuged at 3,000 rpm for 10 min. This extraction buffer was mixed with 0.2% sodium dodecyl sulfate (SDS), 5 mM EDTA, 2.5 ml of phenol previously equilibrated with 10 mM Tris (pH 8), 10 mM NaCl, 1 mM EDTA, and 2.5 ml of chloroform. Aqueous layer was separated by centrifugation and reextracted with chloroform and isoamyl alcohol. RNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol. The solution was placed overnight at –20°C and then centrifuged at 10,000 rpm for 15 min. The resulting RNA pellet was washed with 70% ethanol, air dried, and resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA. The RNA content was quantitated by the absorb-

ance at 260 nm and the degree of protein concentration was determined by the absorbance at 280 nm.

Dot blot hybridization: Heat denatured RNA samples were blotted onto nylon membranes. Membranes were prehybridized in a solution containing 50% deionized formamide, 1x Denhardt's solution, 10% dextran sulfate, 10 mM Tris-HCl, 1 mM EDTA, 600 mM NaCl, 0.5 $\mu\text{g/ml}$ yeast t-RNA and 0.25 mg/ml sonicated salmon sperm DNA at 42°C for 2 hr. Human IL-1 α cDNA probes (1.6 Kb, Eco RI to Hind III fragment containing prepeptide region) [9, 10] kindly provided from Dr. M. Yamada were labeled by photobiotin [8] and hybridized to membranes overnight at 42°C. Membranes were washed 3 times in 2x standard saline citrate (SSC, 1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at 24°C, 3 times in 2xSSC and 0.1% SDS at 65°C and twice in 2xSSC at 25°C. The dots were visualized with the use of streptavidin biotinylated peroxidase complex (DETEK1-hrp, Enzo Biochem., NY, U.S.A.).

Tissues: Necropsied tissue specimens (brain, eye, liver, spleen, and pancreas) were collected from a cat spontaneously infected with FIP virus. Tissues were fixed in 4% paraformaldehyde in PBS for 6 hr at 4°C. They were rinsed 3 times with cold PBS treated with 0.01% diethylpyrocarbonate (Sigma, St Louis, MO, U.S.A.), dehydrated, embedded in paraffin, and sectioned at 4 μm for ISH. Slides were heated at 180°C for 3 hr to eliminate RNase, and coated with 0.001–0.1% poly-L-lysine (Product No. P8920, Sigma St Louis, MO, U.S.A.).

ISH: Sections which had been deparaffinized and rehydrated by routine method were digested with proteinase K (1–100 $\mu\text{g/ml}$) (Sigma, St Louis, MO, U.S.A.) for 10 to 30 min at 37°C, and then treated with 0.3% H_2O_2 in methanol for 30 min at 25°C to block endogenous peroxidase. Each section was treated with 20 to 50 μl of hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 5xSSC, 300 $\mu\text{g/ml}$ of salmon testis DNA and 200–400 $\mu\text{g/ml}$ of biotinylated probe DNA. Plastic lids were applied and sealed with vinyl tape. Each slide was covered with a plastic bag, sealed, and submerged in the water bath. Slides were then transferred to 4 to 37°C incubator and hybridized for various times. After hybridization, slides were treated with 5 min wash with 2xSSC at 25°C, 10 min wash with 2xSSC at 37°C, 5 min wash with 2xSSC at 25°C, 2 min wash with 0.1% Triton X-100 (Sigma, St Louis, MO, U.S.A.) in PBS, and finally rinsed with

PBS. Detection was carried out with the streptavidin biotinylated peroxidase complex (DETEK1-hrp, Enzo Biochem., NY, U.S.A.). The sections were counterstained with methylgreen. The slides were covered with coverslide, and examined with a light microscope. In control experiments, before hybridization sections were treated with 50 $\mu\text{g/ml}$ RNase A (Sigma, St Louis, MO, U.S.A.) and 1 $\mu\text{g/ml}$ RNase T₁ (Sigma, St Louis, MO, U.S.A.) in 2xSSC for 30 min at 37°C.

RESULTS

The homology between human IL-1 α cDNA and feline total mRNA by dot blot hybridization was shown in Fig. 1. The biotinylated human IL-1 α cDNA probe hybridized with only feline total mRNA prepared from LPS-stimulated PM at the concentration of 10 $\mu\text{g/ml}$, although it did not hybridize with that prepared from non-stimulated cells at the same concentration, indicating that feline IL-1 α mRNA may have detectable homology with human IL-1 α cDNA.

The effects of poly-L-lysine coating combined with heat treatment or of heat treatment alone on the application of paraffin-embedded sections to RNase free slide glass were summarized in Table 1. Although slides uncoated and heated at 37°C for 24 hr were poor in the adhesion of sections, slides uncoated and heated at 60°C for 6 hr were better in the adhesion of tissue sections with more than 70% enhancement. Coating with poly-L-lysine was much more effective in adhesion of sections, especially with 0.1% or 0.01% poly-L-lysine and heated at 60°C for 6 hr or longer.

The results of digestion with proteinase K at a variety of concentrations and times at 37°C were summarized in Table 2. Weak or inconsistent reaction could be detected after digestion of proteinase K at the concentration of 1 or 5 $\mu\text{g/ml}$ for 10 to 30 min (Fig. 2A). The proteinase K solution at the concentration of 10 $\mu\text{g/ml}$ gave the same result after 10 min of digestion as 20 min. On the other hand, when proteinase K solution was applied at the concentration of 10 $\mu\text{g/ml}$, the good result was obtained after 30 min of digestion. When proteinase K solution of 50 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ was used, a strong signal was detected in the cytoplasm (Fig. 2B).

The results of denaturation of probes performed at variety of temperature and time were presented in

Fig. 1. A) Dot blot hybridization of RNA from feline peritoneal macrophages. Total RNA were extracted from peritoneal macrophages stimulated with or without LPS (1 µg/ml), and hybridized with human IL-1α probe. There is weak hybridization signal at the concentration of 10 µg/ml of total RNA prepared from LPS stimulated macrophages (arrow).

B) Schematic figure of the resolution of dot blot hybridization. Open circle (○) and closed circle (●) indicate negative hybridization and positive hybridization, respectively.

Table 1. Effect of temperature and time on section adhesion to poly-L-lysine coated and uncoated slides

Treatment	% of poly-L-lysine			
	0	0.001	0.01	0.1
Untreated	— ^{a)}	+ ^{c)}	+	++ ^{d)}
air dry at 25°C				
Heating				
at 37°C for 6 hours	—	+	+	++
at 37°C for 12 hours	+/- ^{b)}	+	+	++
at 37°C for 24 hours	+/-	+	+	++
at 60°C for 6 hours	+	+	++	++
at 60°C for 12 hours	+	+	++	++
at 60°C for 24 hours	+	+	++	++

a) — = No sections remained on slide throughout the ISH procedure.

b) +/- = Less than 70% sections on slide throughout the ISH procedure.

c) += Up to 70% sections remained on slide throughout the ISH procedure.

d) +++ = 100% sections remained on slide throughout the ISH procedure.

Table 2. Effect of proteinase K digestion at 37°C on the ISH

Time	Proteinase K (µg/ml)				
	1	5	10	50	100
10 minutes	+/- ^{a)}	+/-	+/-	+ ^{b)}	+
20 minutes	+/-	+/-	+/-	+	+
30 minutes	+/-	+/-	+	+	+

a) +/- = Weak or inconsistent reaction.

b) += Moderate to strong reaction.

Table 3. No reactions were detected after the denaturation at 40°C and 50°C, and the effect of denaturation on the reaction increased at 60°C, but it was not consistent. The denaturation at 70°C or higher temperature induced a significant increase and consistent reaction in the hybridized signal (Fig. 3). However, the treatment at temperature above 70°C did not enhance the hybridized signal.

The results on necropsied section showed that intense signal was detected after hybridization at 4°C for 24 hr, at 25°C for 18 hr or longer, and 37°C for 5 hr or longer (Table 4). Weak hybridization signals were observed after hybridization at 4°C for 5 or 18 hr, at 25°C for 3 or 5 hr and 37°C for 3 hr. No reactions could be detected after hybridization at 4°C, 25°C and 37°C for 1 hr, or at 4°C for 3 hr. RNase pretreatment before hybridization reduced the hybridization signal or abolished the signal completely (Figs. 4 and 5).

DISCUSSION

ISH techniques using non-radioactive probe have been used for the detection of specific mRNA in routinely processed tissues of the human or murine specimens in the pathology [12, 14, 16, 18, 19]. This ISH technique was introduced to analyze the necropsied specimens. To our knowledge this is the first paper dealing with the detection of the mRNA of

Fig. 2. A) Necropsied pancreatic section from a cat with FIP was treated with proteinase K at the concentration of 5 $\mu\text{g/ml}$ for 30 min. Weak hybridization signal can be seen in the foamy cell (arrow). ($\times 160$)

B) Necropsied pancreatic section from a cat with FIP was treated with proteinase K at the concentration of 100 $\mu\text{g/ml}$ for 30 min. Strong cytoplasmic hybridization signals can be seen in the foamy cells. ($\times 400$)

Table 3. Effect of denaturation of probes on the ISH

Time	40	50	60	70	80	90 ($^{\circ}\text{C}$)
5 minutes	— ^{a)}	—	+/- ^{b)}	+ ^{c)}	+	+
10 minutes	—	—	+/-	+	+	+
15 minutes	—	—	+/-	+	+	+

a) — = Negative reaction.

b) +/- = Weak or inconsistent reaction.

c) + = Moderate to strong reaction.

Table 4. Effect of temperature and time on the hybridization of ISH

Time	4	25	37 ($^{\circ}\text{C}$)
1 hour	— ^{a)}	—	—
3 hours	—	+/- ^{b)}	+/-
5 hours	+/-	+/-	+ ^{c)}
18 hours	+/-	+	+
24 hours	+	+	+

a) — = Negative reaction.

b) +/- = Weak or inconsistent reaction.

c) + = Moderate to strong reaction.

IL-1 α in sections of necropsied tissues in veterinary field.

Initially, homology between human and feline IL-1 α was confirmed by the reaction with the human IL-1 α probe in feline total RNA at the concentration of 10 $\mu\text{g/ml}$ after dot blot hybridization, though the hybridization was very weak. However, the

Fig. 3. Pancreatic section with severe granulomatous and fibronectrotic reaction. The pancreas after denaturation at the temperature of 70 $^{\circ}\text{C}$ for 15 min showing a positive reaction in the mesothelial cell. ($\times 400$)

expression of IL-1 α mRNA in LPS activated feline PM might be low in level, as Oppenheim *et al.* [15] reported that expression of IL-1 α mRNA in the human monocytes treated with LPS achieved a level 2- to 3-fold over that of unstimulated monocytes. To exclude the possibility of non-specific reaction, we

Fig. 4. Necropsied palpebral conjunctiva section from a cat with FIP was hybridized with biotinylated probe without RNase pretreatment. Positive cells are localized in the epithelium layer. ($\times 40$)

Fig. 5. Serial section of Fig. 4. There is no hybridization signal in the serial section treated by same procedure following RNase pretreatment. ($\times 40$)

further checked the homology by means of dot blot analysis using the radioactive probe. Human IL-1 α probe labeled with ^{32}P also detected a spot which applied feline total RNA at the concentration of 10 $\mu\text{g/ml}$ (data not shown). These results indicated that human IL-1 α cDNA had a homology with feline IL-1 α mRNA.

We explored some modification in ISH procedures reported by Hondo *et al.* [13]. At the first step, we examined the effect of coating on section adhesion to the glass slide. Our results showed that coating slides with poly-L-lysine and heating at 37°C to 60°C at least for 6 hr gave good results in adhesion of sections. Heat treatment was also effective, though the percent of section loss was higher on slides treated with heat alone than that on slides treated with poly-L-lysine and heat. There was no significant difference between the treatment with poly-L-lysine and heat in the adhesion of sections on the slide treated as follows. After sections were mounted on a RNase free slide, the slide with sections was completely dried as fast as possible by heat treatment (50–52°C) on the hot plate and then were heated in the oven.

The effect of enzyme digestion of ISH was examined. Although the hybridization signal was very weak at a low concentration of proteinase K (Fig. 2A, Table 2), a consistent reaction was obtained at higher concentrations, 50 $\mu\text{g/ml}$ or

greater (Fig. 2B, Table 2). Treatments with high concentration of proteinase K solution for the digestion in shorter or longer time did not improve further. While, we could not find the destruction of cytological structures and the increase of background staining on sections which were treated with 10 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ solution of proteinase K. However, Angerer and Angerer [1] reported that optimal conditions for proteinase K digestion to get maximum hybridization signal without destruction of cytological structures of tissues were narrow limits. In addition, Hankin and Lloyd [12] showed that proteinase K treatment at a high concentration increased background staining. Such differences might be originated from the condition of tissue fixation and the susceptibility to proteinase K solution [16, 22]. Our results of denaturation and hybridization conditions on the signal in this experiment revealed that denaturation for 5 min at least at 70°C or higher and overnight hybridization at 25 or 37°C produced consistent reaction. These conditions were not consistent with those reported previously [3, 5, 20, 21], showing that optimal conditions should be determined in each experiment.

The signal was detectable in some specific cells including macrophages, but not in other cells such as hepatocytes, suggesting that signals detected by our method are specific to IL-1 α mRNA [6, 15, 18]. Moreover, the specificity of the signal for IL-1 α mRNA was confirmed by Northern hybridization

and RNase treatment.

This report presents that a biotinylated cDNA probe can be used to detect specific mRNA in paraffin-embedded tissue sections in cats. This ISH technique should be very useful for detecting specific mRNA in the tissue sections and may give a detailed information of pathogenesis in animal diseases.

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