EXPRESSION OF INTERLEUKIN 1α mRNA IN FELINE INFECTIOUS PERITONITIS

（ネコ伝染性腹膜炎におけるインターロイキン1α mRNAの発現に関する研究）

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CONTENT

SUMMARY

Although it is well known that all serous surfaces throughout the body of cats affected with feline infectious peritonitis (FIP) are involved in the inflammatory response, the role of macrophage and macrophage-derived cytokines have not been clarified. Recently, it has been shown that not only IL-1ß but also IL-1α is indispensable in regulation of inflammatory and immune responses. Therefore, the role of IL-1α in inflammatory response of cats with FIP was examined by *in situ* hybridization (ISH) with biotinylated probes.

ISH technique with biotin-labeled probes was employed for detecting feline IL-1 mRNA, especially IL-1α mRNA, in paraffin-embedded sections from autopsied specimens of FIP. Homology between human IL-1α cDNA used as a probe and feline IL-1α mRNA was confirmed by means of dot blot hybridization using the biotin-labeled probe. The following conditions were found to be the best as a routine procedure; 1)coating of slides with poly-l-lysine and/or heating at 60°C at least for 6 hours gave an excellent condition for the adhesion of tissue sections, 2)proteinase K solution (50 to 100 μg/ml) treatment for 20 to 30 minutes at 37°C gave the best results in the detection of ISH signal, 3)suitable denaturation time at 70 to 80°C was 10 to 15 minutes, and 4)effective hybridization was obtained by incubation for 18 to 24 hours at 37°C.

By this improved ISH procedure with biotinylated human IL-1α cDNA probes,

the tissue distribution of feline IL-1α mRNA-expressing cells was examined in 3 cases of healthy cats. Feline IL-1α mRNA-positive cells were detected in the brain, lymphoid organs and liver, although the number of IL-1α mRNA-expressing cells was few. In the brain, hybridization signals were found in the cytoplasm of a small number of vascular endothelium and anterior pituitary cells. In lymphoid organs, reticuloendothelial cells and macrophages contained IL-1α mRNA. In the liver, hybridization signals were observed in Kupffer cells. These findings suggest that IL-1α may relate to the regulation of immune reaction against incessant invading antigens.

By ISH with biotinylated human IL-1α cDNA probes, the tissue distribution of feline IL-1α mRNA-expressing cells was examined in 49 cases diagnosed as FIP in the past 10 years. All tissue specimens consisted of routinely processed paraffin blocks. Feline IL-1α mRNA was detectable in the specimens from blocks prepared in the past 10 years, suggesting that fixed RNA may be stable in the paraffin-embedded tissues for at least 10 years. In cats affected with FIP, a large number of cells expressing IL-1α mRNA were found in the cellular infiltrations on the visceral peritoneum including omentum and/or serosal surface of various organs. The morphology of these cells indicated that these positive cells were macrophages or infiltrated mononuclear cells. In non-lesional area of these organs, the distribution of IL-1α mRNA-producing cells in cats with FIP was not significantly different from that in healthy cats. These findings support the concept that IL-1α as well as immune complexes is involved in the development of inflammatory response of FIP.

IL-1α might be produced by macrophages in the local inflammatory sites at the early phase of host reaction and should participate in the initiation and/or development of the inflammatory response.

PROLOGUE

Feline infectious peritonitis (FIP) is a coronavirus infection of cats resulting in chronic and progressive disease. In general, FIP can be divided into two forms. One is effusive "wet" form in which the fibronecrotic, exudative inflammation of the serosae prevails and the other is noneffusive "dry" form in which the small granulomatous lesions in parenchymal organs are prominent with no peritoneal and/or pleural effusion. The basic histopathological lesion of FIP is a vasculitis and focal pyogranulomatous reaction in the visceral peritoneum and parenchymatous organs. Previous experiments have demonstrated large amounts of FIP virus (FIPV) in macrophages located at the periphery of perivascular pyogranulomatous lesions in FIPV-infected cats. Besides, complexes of immunoglobulin G and the third component of complement bound specifically to FIPV were demonstrated in the cytoplasm of infected macrophages. These findings indicate that immune mediated mechanism is involved in the pathogenesis of FIP. In another words, the lesions of FIP are caused by the deposition of FIPV antigen-antibody complexes that fix complement leading to Arthus-type reaction. On the other hand, it is well known that continuous inflammatory response can be observed in the lesions of FIP. Generally, it is thought that immune complexes described above induce inflammatory response including continuous inflammation of FIP, although the pathogenesis of inflammatory response on the visceral peritoneum remains obscure. Since

macrophage is a target cell of FIPV, it is suggested that macrophage and macrophage-derived cytokines such as IL-1 participate in the inflammatory response in FIP.

Recently, it has been shown that macrophage and macrophage-derived cytokines such as interleukin 1 (IL-1) play a key role in regulation of inflammatory response. IL-1, which is produced mainly by mononuclear phagocytes, is an important cytokines in the regulation of immune response. Two distinct IL-1, IL-1α (pI:5.0) and IL-1ß (pI:7.0), have been molecularly cloned from human, mouse and rabbit. These 2 kinds of IL-1 have similar biological activities and share the same receptor on the target cells, indicating that IL-1α and IL-1ß react almost the same *in vivo*. Since IL-1α is not a dominant type of IL-1, it was thought that the activity of IL-1α in immune response is lower than that of IL-1ß. Recent findings suggest that IL-1α contribute to the initiation and/or regulation of immune and inflammatory responses as well as IL-1ß. The role of IL-1α in the inflammatory response of FIP, however, has not been clarified. To disclose the role of IL-1α in the inflammatory response of FIP, this study was carried out on the tissue distribution of cells expressing IL-1α mRNA in cats with FIP by *in situ* hybridization (ISH) with biotinylated probes. In chapter 1, the condition of ISH procedure with biotinylated probes was examined in detail to detect feline IL-1α mRNA. In chapter 2 and 3, the tissue distributions of feline IL-1α mRNA-expressing cells were shown in healthy cats and FIP affected cats by ISH technique, respectively, with the discussion on the role of IL-1α in the inflammatory response of FIP.

CHAPTER 1

IN SITU HYBRIDIZATION FOR THE DETECTION OF FELINE INTERLEUKIN 1α mRNA ON THE PARAFFIN-EMBEDDED SECTIONS USING BIOTIN-LABELED PROBES

ABSTRACT

In situ hybridization (ISH) technique with biotin-labeled probes was employed for detecting feline interleukin 1 (IL-1) mRNA, especially IL-1α mRNA, in paraffin-embedded sections from autopsied specimens of feline infectious peritonitis (FIP). Homology between human IL-1α cDNA used as a probe and feline IL-1α mRNA was confirmed by means of dot blot hybridization using the biotin-labeled probe. The following conditions were found to be best as routine procedures; 1)coating of slides with poly-l-lysine and/or heating at 60°C at least for 6 hours gave an excellent condition for the adhesion of tissue sections, 2)proteinase K solution (50 to 100 μg/ml) treatment for 20 to 30 minutes at 37°C gave the best results in the detection of ISH signal, 3)suitable denaturation time at 70 to 80°C was 10 to 15 minutes, and 4)effective hybridization was obtained by incubation for 18 to 24 hours at 37°C.

INTRODUCTION

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 (5,11,19,28,30,38,48,49,55,56). 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 (5,28,30,38,56). In the present study, the author attempted to apply the ISH procedure with a biotinylated probe for interleukin 1α (IL-1α) to paraffin-embedded sections from a cat with feline infectious peritonitis (FIP) in which the overproduction of IL-1 had been reported (30).

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, MICHIGAN, USA). PM were harvested by sterilely 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 μg/ml of lipopolysaccharide (LPS) (SIGMA, ST LOUIS, MO, USA) in tissue culture medium, chilled on ice, scraped, and transferred to a sterile tube. These cells were centrifuged at 1000 rpm for 7 min, and the precipitated cells were suspended in 2 ml of medium. LPS-treated and non-treated PM were flooded with 5 ml of NP40 lysis buffer containing 1.4mM NaCl, 0.015mM $MgCl_2$, 0.1mM Tris (pH 8.6) and 5% ribonucleoside vanadyl complexes (SIGMA, ST LOUIS, MO, USA), incubated for 10 min at 4°C, added with 125μl of 20% Nonidet p40 (SIGMA, ST LOUIS, MO, USA), and then centrifuged at 3000 rpm for 10 min. This extraction buffer was mixed with 0.2% sodium dodecyl sulfate (SDS), 5mM EDTA, 2.5 ml of phenol previously equilibrated with 10mM Tris (pH 8), 10mM NaCl, 1mM EDTA, and 2.5 ml of chloroform. Aqueous layers was separated by centrifugation and

reextracted with chloroform and isoamyl alcohol. RNA was precipitated by adding 1/10 volume of 3M sodium acetate and 2.5 volume of ethylalcohol. The solution was placed overnight at -20°C and then centrifuged at 10000 rpm for 15 min. The resulting RNA pellet was washed with 70% ethanol, air dried, and resuspended in 10mM Tris (pH 7.4) with 1mM EDTA. The RNA content was quantitated by the absorbance 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, 10mM Tris-HCl, 1mM EDTA, 600mM NaCl, 0.5 ng/ml yeast t-RNA and 0.25 mg/ml sonicated salmon sperm DNA at 42°C for 2 hr. Human IL-1α cDNA probes which was kindly provided from Dr. M. Yamada (DAINIPPON PHARMACEUTICALS, OSAKA, JAPAN) were labeled by photobiotin (22) and hybridized to membranes overnight at 42°C. Membranes were washed 3 times in 2x standard saline citrate(SSC, 1xSSC is 0.15M NaCl plus 0.015M sodium citrate) and 0.1% SDS at 25°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 BIOCEM., NY, USA).

Tissues: Specimens 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, USA), dehydrated, embedded in paraffin, and sectioned at 4 μm for ISH. Slides were heated at 180°C for 3 hr to eliminate RNase.

ISH: Sections which had been deparafinized and rehydrate by routine method were digested with proteinase K (1-100 μg/ml) (SIGMA, ST LOUIS, MO, USA) for 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 μg/ml of salmon testis DNA and 200 - 400 ng/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, USA) in PBS, and finally rinsed with PBS. Detection was carried out with the streptavidin biotinylated peroxidase complex (DETEK1-hrp, ENZO BIOCEM., NY, USA). The sections were counterstained with methylgreen. The slides were covered with coverslides, and examined with a light microscope. In control experiments, before hybridization sections were treated with 50 μg/ml RNase A (SIGMA, ST LOUIS, MO, USA) and 1 μg/ml RNase T_1 (SIGMA, ST LOUIS, MO, USA) in 2xSSC for 30 min at 37°C.

RESULTS

The homology between human IL-1α cDNA and feline IL-1α mRNA by dot blot hybridization was shown in Figure 1. The biotinylated human IL-1α cDNA probe hybridized with mRNA in LPS-stimulated feline PM, but not with that in non-stimulated cells, indicating that feline IL-1α mRNA had detectable homology with human IL-1α cDNA.

The optimal hybridization condition was as follows. 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 section 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 a concentration of 1 or 5 μg/ml for 10 to 30 min (Fig. 2A). The proteinase K solution at a concentration of 10 μg/ml gave the same result after 10 min of digestion as to 20 min. On the other hand, when proteinase K solution was applied at the concentration of 10 μg/ml, a good result

was obtained after 30 min of digestion. When proteinase K solution of 50 μg/ml or 100 μg/ml was used, a strong signal was detected in the cytoplasm (Fig. 2B).

The results of denaturation performed at variety of temperature and times were presented in 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 the temperature above 70°C did not enhance the hybridized signal.

The results on autopsied sections 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 (Fig. 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 (28,33,48,55,56). This ISH technique was introduced to analyze the autopsied specimens. To my knowledge this is the first paper dealing with the detection of the mRNA of IL-1α in sections of autopsied tissues in veterinary field.

Initially, homology between human and feline IL-1α was confirmed. The human IL-1α probe have only a weak reaction in feline total RNA after dot blot hybridization. Since the hybridization was very weak, to exclude the possibility of non-specific reaction, the homology was checked by dot blot analysis using the radioactive probe. IL-1α probe labeled with ^{32}P also detected a spot which applied total RNA at the concentration of 10 μg/ml (data not shown). These results indicated that human IL-1α cDNA had a homology with feline IL-1α mRNA. However, the expression of IL-1α mRNA in LPS activated feline PM might be low in level, as Oppenheim *et al.* (43) reported that expression of IL-1α mRNA in the human monocytes treated with LPS achieved a level 2- to 3-fold over that of unstimulated monocytes.

Some modification was explored in ISH procedures reported by Hondo *et al.* (30). At the first step, the effect of coating on section adhesion was examined. The results showed that coating slides with poly-l-lysine and heating at 37°C to 60°C at

least for 6 hr gave good conditions for adhesion of sections. Heat treatment was also effective. The percent of section loss was higher on slides treated with heat alone than that on slides treated with poly-l-lysine and heat. Almost all the sections remained on slides when treated as follows. After sections were mounted on 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. There was no significant difference between the treatment with poly-l-lysine and heat in the adhesion of sections on a slide. Subsequently, the effect of enzyme digestion on ISH was examined. Although the hybridization signal was very weak at low concentration of proteinase K (Fig. 2A, Table 2), a consistent reaction was obtained at higher concentrations, 50 μg/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, the author could not find the destruction of cytological structures and the increase of background staining on sections which were treated with 10 μg/ml to 100 μg/ml solution of proteinase K. However, Angerer *et al.* (3) reported that optimal conditions for proteinase K digestion to get maximum hybridization signal without destruction of cytological structures of tissues were in narrow limits. In addition, Hankin *et al.* (28) 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 digestion (48,64). The results of denaturation and hybridization conditions on the signal in this experiments 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 (8,12,57,61), showing that optimal conditions should be determined in each experiment.

The signal was detectable in the some of specific cells including macrophages, but not in other cells such as hepatocytes, suggesting that signals detected by this method are specific to IL-1α mRNA (16-19,42,43,55). Moreover, the specificity of the signal for IL-1α mRNA was confirmed by northern hybridization and RNase treatment.

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

CHAPTER 2

DISTRIBUTION OF INTERLEUKIN 1α (IL-1α) mRNA-EXPRESSING CELLS IN HEALTHY CATS

ABSTRACT

Distribution of feline interleukin 1α (IL-1α) mRNA-expressing cells in the tissue of 3 cases of healthy cats was examined by *in situ* hybridization with biotinylated probe. Feline IL-1α mRNA-positive cells were detected in the brain, lymphoid organs and liver, although they were few in number. In the brain, hybridization signals were found in the cytoplasm of a small number of vascular endothelium and anterior pituitary cells. In lymphoid organs, reticuloendothelial cells and macrophages contained IL-1α mRNA. Hybridization signals were also observed in Kupffer cells in the liver. These findings suggest that IL-1α may relate to the regulation of immune reaction against incessant invading antigens.

INTRODUCTION

Interleukin 1 (IL-1) is one of the important cytokines in the biological response to foreign antigens (16-18,42-44). Recently, two distinct IL-1, IL-1α (pI:5.0) and IL-1ß (pI: 7.0), have been molecularly cloned from human, mouse and rabbit (23,27,39), and attention has been focused on the role of IL-1α in the regulation of immune response to foreign antigens (15,21,45,58). IL-1 including α and ß form is produced by a variety of cells such as monocytes, macrophages, fibroblasts, chondrocytes and neutrophils (16,17,42,43). Though these cellular sources have been determined by *in vitro* study, detailed information on the production of IL-1α *in vivo* is lacking. Therefore, the tissue distribution of cells expressing IL-1α mRNA in different organs of healthy cats was examined by *in situ* hybridization (ISH) with biotinylated probes.

MATERIALS AND METHODS

Preparation of biotinylated probes: Human IL-1α cDNA probe (1.6 Kb, GC: 36%) which were kindly provided from Dr. M. Yamada (DAINIPPON PHARMACEUTICALS, OSAKA, JAPAN) were labeled by photobiotin (22). Autoclaved (121°C, 60 min) ultra-pure water was treated with 0.01% diethylpyrocarbonate (DEPC) (SIGMA, ST LOUIS, MO, USA) to remove contaminating RNases. This DEPC treated water was used throughout this experiments.

Preparation of tissues and sections: Clean slides were coated with 0.01% poly-l-lysine (SIGMA, ST LOUIS, MO, USA) and then heated at 180°C for 3 hr to remove contaminating RNases (29). Autopsied samples of healthy cats were taken from 3 healthy cats (FIP titer < 1/160). They were routinely fixed in neutral buffered formalin (pH 7.2) for 48 to 72 hr, dehydrated, embedded in paraffin, sectioned at 4 μm for ISH. Slides with section were heated at 60°C for 12 hr (29).

ISH: Feline IL-1α mRNA was stained by a method as previously described (29). In brief, sections which had been deparafinized and rehydrated with RNase-free reagents treated by DEPC were digested with proteinase K (SIGMA, ST LOUIS, MO, USA) (50 μg/ml) for 30 min at 37°C, and then treated with 0.3% H_2O_2 in methanol for 30 min at 25°C. Each individual section was incubated with 20 to 50 μl of hybridization buffer containing 50% deionized formamide, 10% dextran sulfate,

5x standard saline citrate(SSC, 1xSSC is 0.15M NaCl plus 0.015M sodium citrate), 300 μg/ml of salmon testis DNA and 200 - 400 ng/ml of biotinylated probe DNA. Slides were incubated at 80°C for 15 min within a moist box, and subsequently hybridized at 37°C for 18 hr. 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, USA) in phosphate buffered saline (PBS, pH 7.4) and finally rinsed with PBS. Detection was carried out with the streptavidin biotinylated peroxidase complex (DETEK1-hrp, ENZO BIOCEM., NY, USA). Sections were counterstained with methylgreen, covered with coverslide and examined with a light microscope. As a control experiment, before hybridization serial sections were treated with 50 μg/ml RNase A (SIGMA, ST LOUIS, MO, USA) and 1 μg/ml RNase T_1 (SIGMA, ST LOUIS, MO, USA) in 2xSSC for 30 min at 37°C and hybridized by a same procedure as described above.

RESULTS

Since the procedure of fixation in this study was quite different from that in chapter 1, the preliminary experiments were performed in order to confirm the optimal condition of ISH. It was revealed that the optimal condition was almost the same as described in chapter 1. However, the condition of section adhesion was preferable when slide coated with 0.01% poly-l-lysine were heated at 60°C for 12 hr after applying the sections.

In healthy cats, a small number of IL-1α mRNA-expressing cells were detected in the brain, liver and lymphoid organs such as the spleen and lymph nodes as shown in Table 5. In the brain, hybridization signals were found in the cytoplasm of vascular endothelium (Fig. 6A). In addition, IL-1α mRNA-positive cells were seen in the anterior pituitary (Fig. 6B). Positive hybridization was associated with Kupffer cells in the liver (Fig. 7). In the spleen, IL-1α mRNA-containing cells were located at the red pulp (Fig. 8A, 8B). In lymph nodes, hybridization signals were found in the sinuses (Fig. 9). The morphology and the localization of positive cells of lymphoid organs were similar to that of reticuloendothelial cells or macrophages. Cells containing IL-1α mRNA were not detectable in the other organs (Table 5).

Positive hybridization signals were abolished by RNase treatment before hybridization (Fig. 6B and 6C).

DISCUSSION

In the present study, the distribution of feline IL-1α mRNA-positive cells in healthy cats was demonstrated by ISH with biotinylated DNA probes. Optimal condition of ISH was consistent with chapter 1, except section application (29). Since heat treatment did not give the good adhesion, the procedure of poly-l-lysine coating was applied combining with heat for the section adhesion.

Hybridization signal on the section, as expected, was detectable by means of this method. However, IL-1α mRNA-containing cells could not be found in many organs as compared to previous report (55). This may be due to the different system for the detection of specific mRNA (28) and/or the dominancy of feline IL-1α (16,17,29,42, 43).

In healthy cats, few IL-1α mRNA-containing cells were demonstrated in tissues such as brain, liver and lymphoid organs, although they were not in the other organs (Table 5). Interestingly, IL-1α mRNA-expressing cells were found in the brain, because the recent findings suggest that IL-1 which is present in the brain serve as a neuromodulator including neuroendocrine function of the acute phase reaction in central nervous system (6,9,44,53,62). In this experiments, IL-1α mRNA was found in the vascular endothelium and the pituitary. Owing to presence of blood-brain barrier, plasma IL-1 can not directly enter the cerebral parenchyma. Hence, IL-1 derived from vascular endothelium or pituitary may influence neuroendocrine

functions (44,62). IL-1α mRNA-expressing cells in the lymphoid organs and liver were reticuloendothelial cells or macrophages and Kupffer cells, respectively. Consistent with our observation, Takács *et al.* (55) have demonstrated that IL-1 producing cells, which are almost tissue macrophages, in normal mice are generally localized in lymphoid organs and liver exposed for antigen. These findings indicate that IL-1α is mainly produced by a small number of activated cells of reticulo-endothelial system including macrophages, when they are exposed to antigens. IL-1α may relate to the regulation of immune response including central nervous system against incessant invading antigens.

CHAPTER 3

EXPRESSION OF INTERLEUKIN 1α (IL-1α) mRNA IN CATS WITH FELINE INFECTIOUS PERITONITIS

ABSTRACT

By *in situ* hybridization (ISH) with biotinylated human interleukin 1α (IL-1α) cDNA probe, tissue distribution of feline IL-1α mRNA-expressing cells was examined in 49 cases diagnosed as feline infectious peritonitis (FIP) in the past 10 years. All tissue specimens examined consisted of routinely processed paraffin blocks of these cases. Feline IL-1α mRNA detected in these blocks suggested that fixed RNA was stable in the paraffin-embedded tissues for at least 10 years. In cats affected with FIP, a large number of cells expressing IL-1α mRNA were found in the inflammatory lesions on the visceral peritoneum including omentum and/or serosal surface of various organs. The morphology of these positive cells indicated that they were macrophages or infiltrated mononuclear cells. In non-lesional area of these organs, the distribution of IL-1α mRNA-producing cells in cats with FIP was not significantly different from that in healthy cats. These findings support the concept that IL-1α as well as immune complexes is involved in the development of inflammatory response of FIP. IL-1α might be produced by macrophages in the local inflammatory sites at the early phase of host reaction and should participate in the initiation and/or development of the inflammatory response.

INTRODUCTION

Feline infectious peritonitis (FIP) is a coronavirus infection of cats resulting in chronic and progressive disease, and it can be divided into effusive "wet" form and noneffusive "dry" one. In the effusive form of the disease, the macroscopic feature is a visceral and parietal peritonitis covered by a fibrinous exudate which is mainly composed of fibrin and infiltrated cells, such as macrophages, lymphocytes, neutrophils, and fibroblasts (31,32). Generally, continuous inflammatory response can be observed in this lesion. The basic histopathological lesions of FIP are vasculitis and focal pyogranulomatous reaction in the visceral peritoneum and parenchymatous organs. It is thought that the deposition of FIP virus (FIPV) antigen-antibody complexes and complement induce inflammatory response in FIP (31,32,46,59,60), although the pathogenesis of continuous inflammatory response on the visceral peritoneum remains obscure.

Recently, it has been shown that macrophage-derived cytokines such as interleukin 1 (IL-1) regulate the inflammatory response (1,16-18,34,42-44). IL-1α as well as IL-1ß is considered as an important cytokines in immune and inflammatory responses (4,14-17,21,35,37,42-45,58). For instance, IL-1α plays a key role in initiation of immune responses by T lymphocytes through activation of antigen presenting cells (21). Barkley *et al.* (4) also reported that IL-1α might be involved in the pathogenesis of rheumatoid inflammation. Since macrophages are target cells

of FIPV (3,46,59,60), it is suggested that macrophage and macrophage-derived IL-1α deeply participate in the inflammatory response in FIP (16,17,25,34,42-44). Direct evidence of IL-1α on the inflammatory response of FIP, however, is lacking. In the present study, the distribution of IL-1α mRNA-expressing cells was examined in FIP affected cats by *in situ* hybridization (ISH), and the role of IL-1α in the inflammatory response in FIP affected cats was discussed.

MATERIALS AND METHODS

Preparation of biotinylated probes: Human IL-1α cDNA probes (1.6 Kb, GC: 36%) kindly provided by Dr. M. Yamada (DAINIPPON PHARMACEUTICALS, OSAKA, JAPAN) were labeled by photobiotin (22). Autoclaved (121°C, 60 min) ultra-pure water was treated with 0.01% diethylpyrocarbonate (DEPC) (SIGMA, ST LOUIS, MO, USA) to remove contaminating RNases. This DEPC treated water was used throughout this experiments.

Preparation of tissues and sections: Clean slides were coated with 0.01% poly-l-lysine (SIGMA, ST LOUIS, MO, USA) and then heated at 180°C for 3 hr to remove contaminating RNases (29). Autopsied tissue specimens from 49 FIP affected cats were obtained during the past 10 years at the Department of Veterinary Pathology, the University of Tokyo. All specimens routinely processed paraffin blocks were sectioned at 4 μm for ISH. Slides with sections were heated at 60°C for 12 hr (29).

ISH: Feline IL-1α mRNA was stained by a method as previously described (29). In brief, sections which had been deparafinized and rehydrated with RNases-free reagents treated by DEPC were digested with proteinase K (SIGMA, ST LOUIS, MO, USA) (50 μg/ml) for 30 min at 37°C, and then treated with 0.3% H_2O_2 in methanol for 30 min at 25°C. Each individual section was incubated with 20 to 50 μl of hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 5x standard saline citrate(SSC, 1xSSC is 0.15M NaCl plus 0.015M sodium citrate),

300 μg/ml of salmon testis DNA and 200 - 400 ng/ml of biotinylated probe DNA. Slides were incubated at 80°C for 15 min within a moist box, and subsequently hybridized at 37°C for 18 hr. 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, USA) in phosphate buffered saline (PBS, pH 7.4) and finally rinsed with PBS. Detection was carried out with the streptavidin biotinylated peroxidase complex (DETEK1-hrp, ENZO BIOCEM., NY, USA). Sections were counterstained with methylgreen, covered with coverslide and examined with a light microscope. As a control experiment, before hybridization serial sections were treated with 50 μg/ml RNase A (SIGMA, ST LOUIS, MO, USA) and 1 μg/ml RNase T_1 (SIGMA, ST LOUIS, MO, USA) in 2xSSC for 30 min at 37°C and hybridized by a same procedure as described above.

RESULTS

By ISH procedure described in chapter 2, hybridization signals of IL-1α mRNA was detected on the formalin-fixed and paraffin-embedded sections of almost all cases examined (Table 6).

Table 7 summarizes the tissue distribution of IL-1α mRNA-expressing cells in cats with FIP. In general, most of IL-1α mRNA-positive cells were localized in the fibrinous and cellular exudate on the serosa of various organs. Many cells showing IL-1α mRNA were found in the mononuclear cells infiltrated on the visceral peritoneum including the serosa (Fig. 10A-C). Furthermore, the aggregation of positive cells was also observed in the cellular infiltrations on the omentum and peritoneum (Fig. 10C and 10D). IL-1α positive cells, however, were not detectable in the matured lesions (Fig. 10C). Morphological observation suggested that these cells were macrophages or monocytes. No hybridization signals were found in the cytoplasm of fibroblasts (Fig. 10A, 10C). Infrequent positive hybridization was associated with vascular endothelial cells in the omentum with a thick layer of fibrin adherent and severe inflammatory response (Fig. 11). Few IL-1α mRNA-expressing cells were found in the liver, spleen, lymph nodes, kidney, pancreas, intestinal tract, pleura, brain, palpebral conjunctiva and bone marrow. In the liver, hybridization signals were localized in the cytoplasm of Kupffer cells (Fig. 12). In the lymphoid organs such as spleen and lymph nodes, positive hybridization was associated with

reticuloendothelial cells or macrophages (Fig. 13). IL-1α mRNA-producing cells in the spleen and lymph nodes were localized in the red pulp and sinuses, respectively. Hybridization was observed in a scattered form in the kidney (Fig. 14). Positive infiltrated mononuclear cells were found in the medullary (Fig. 14A) and/or cortical areas (Fig. 14B), whereas no positive cells were observed in the glomeruli (Fig. 14B). In the pancreas with severe granulomatous response, hybridization signals could be seen in the foamy mononuclear cells (Fig. 15). In the intestine, IL-1α mRNA-expressing cells were found in the mucosal layer (Fig. 16A) or muscular layer (Fig. 16B). These cells with hybridization signal were identified morphologically as macrophages or infiltrated mononuclear cells. Very few positive cells could be seen in the visceral pleura (Fig. 17). It seemed that these cells were involved in the effusive pleuritis. In the brain, signals were localized in the cytoplasm of vascular endothelium (Fig. 18A) or mononuclear cell on the ependyma (Fig. 18B). In addition, IL-1α mRNA was found in the ependymocytes (Fig. 18C). There were IL-1α mRNA-containing cells in the epithelium layer at palpebral conjunctiva (Fig. 19). IL-1α mRNA-producing cells were also localized in the bone marrow (Fig. 20).

Positive hybridization signals were abolished in all cases by RNase treatment before hybridization (Fig. 16B and 16C), suggesting that these signals indicate the presence of mRNA.

DISCUSSION

The present study revealed that the hybridization signal-positive cells could be found in cat with FIP by ISH with biotinylated DNA probe (Table 7). Besides, IL-1α mRNA was detectable in tissues which had been collected during the past 10 years (Table 6). Hankin *et al.* (28) also reported that hybridization signal of mRNA from 10-year-old archival paraffin blocks was detectable by ISH with biotinylated probes. These evidences suggest that fixed RNA which had not been degraded by RNase is stable in the paraffin-embedded tissues for at least 10 years.

The hybridization signals were frequently observed in the omentum and serosal surface of various organs in FIP affected cats (Table 7). Most positive cells were localized in the early phase of the inflammatory response, whereas no IL-1α mRNA was found in the late phase of the inflammation (Fig. 10). Indeed, the quality of the staining in the specimens was influenced by; 1)the time from between death and autopsy (3 to 24 hours in this study), 2)the sensitivity of ISH on the each specimen, 3)the amount of feline IL-1α mRNA. Nevertheless, the distribution of IL-1α mRNA-positive cells was quite similar to the pattern in all autopsy specimens. These results suggest that IL-1α may be produced by macrophages in the local inflammatory sites at the early phase of host reaction and may participate in the initiation and development of inflammatory response (1,7,10,13,16,18,20,33,34,41,42-44,47,50,53,54). In the inflammation of FIP, there are at least 2 possible explanations for the role of

IL-1α; 1)IL-1α may play an important part as a chemotactic factor (16,17,41,42-44) and may regulate the production of chemotactic cytokines, such as monocyte chemotactic protein-1 (MCP-1) and neutrophil-activating protein-1/interleukin 8 (NAP-1/IL-8) (16,17,34,36,42-44,52,63), 2)IL-1α may activate mononuclear cells in local inflammatory sites by more secretion of cytokines such as IL-6 and colony stimulating factor (1,2,16-18,26,42-44). While, infrequent hybridization signals were detected in the cytoplasm of vascular endothelium in the omentum and/or visceral peritoneum with severe effusive form (Fig. 11), indicating that IL-1α derived from vascular endothelium might be involved in the pathogenesis of inflammation (7,16-18,41,42-44). In the brain, the number of cells containing IL-1α mRNA in FIP cats was greater than that in healthy control cats, suggesting that IL-1α may activate the neuroendocrine functions to stimulate the immune reaction under the disease (6,9,44,53,62).

On the other hand, few positive cells were found in the chronic inflammatory lesions in the liver, spleen, kidney and lung. In these organs, there was no significant difference on the distribution of IL-1α containing cells between FIP affected cats and healthy controls (Table 5 and 7), indicating that IL-1α might have a minor role in the maintenance of chronic inflammation in FIP (13,33,53). Since most positive cells were macrophages that are target cells of FIP (3,46,59,60), it can be suggested that FIPV infection participates in the induction of IL-1α mRNA of macrophages (16,17, 42-44). Although FIPV is generally observed in the macrophages at the periphery of perivascular lesions (46,59,60), no IL-1α mRNA was found in the lesions of

vasculitis (data not shown), supposing that immune complexes may be principal factor in the development of vasculitis (31,32,46,59,60).

The comprehension of the role of macrophages and macrophage-derived cytokines should help us to understand the pathogenesis of inflammatory response in animals and humans. Furthermore, by detection of mRNA with ISH for other cytokines including IL-1ß, the precise role of cytokines involved in inflammatory response should be clarified.

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LEGEND OF FIGURES

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

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

Table 3: Effect of denaturation on the ISH.

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

Table 5: Feline IL-1α mRNA expression in healthy cats.

Table 6: IL-1α mRNA detected cases with FIP over the period studied.

Table 7: Feline IL-1α mRNA expression in FIP affected cats.

Fig. 1: 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.

Fig. 2: A), Autopsied pancreatic section from a cat with FIP was treated with proteinase K at the concentration of 5 μg/ml for 30 min. Weak hybridization signal can be seen in the foamy cell (arrow). (x 160)

B), Autopsied pancreatic section from a cat with FIP was treated with proteinase K at the concentration of 100 μg/ml for 30 min. Strong cytoplasmic hybridization signals can be seen in the foamy cells. (x 400)

Fig. 3: Pseudomembranous pancreas section. The pancreas after denaturation at the temperature of 70°C for 15 min showing positive reaction in the mesothelial cell. (x 400)

Fig. 4: Autopsied palpebral conjunctiva section from FIP infected cat was hybridized with biotinylated probe without RNase pretreatment. Positive cells are localized in the epithelium layer. (x 40)

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

Fig. 6: *In situ* hybridization analysis of feline IL-1α mRNA expression in brain.

A), The vascular endothelium of the brain contains IL-1α mRNA. (x 100)

B), IL-1α mRNA-expressing cells are found in the pituitary. (x 40)

C), RNase treated serial section of Fig. 6B. IL-1α mRNA can not be seen in the serial section treated by same procedure following RNase pretreatment. (x 40)

Fig. 7: *In situ* hybridization analysis of feline IL-1α mRNA expression in liver. Cytoplasmic hybridization signal can be seen in the Kupffer cell in the liver (arrow). (x 120)

Fig. 8: *In situ* hybridization analysis of feline IL-1α mRNA expression in spleen.

A), Weak cytoplasmic hybridization signal is found in the reticuloendothelial cell in the spleen (arrow). (x 250)

B), High power photomicrograph of IL-1α mRNA-expressing cell. IL-1α mRNA is observed in the cytoplasm of macrophage in the red

pulp of spleen. (x 400)

Fig. 9: *In situ* hybridization analysis of feline IL-1α mRNA expression in lymph nodes. Weak cytoplasmic hybridization signals can be seen in the reticuloendothelial cells in sinuses of lymph nodes (arrows). (x 250)

Fig. 10: *In situ* hybridization analysis of feline IL-1α mRNA expression on the visceral peritoneum including the serosa and omentum of FIP affected cats.

A), Higher number of IL-1α mRNA-expressing cells is localized in the fibrinous and cellular exudate on the serosa of mesenteric lymph node. IL-1α mRNA is in the cytoplasm of macrophage-like mononuclear cells, but not fibroblasts. (x 40)

B), High power photomicrograph of positive cells on the omentum. Several large, fused macrophage-like cells contain IL-1α mRNA. (x 160)

C), Several IL-1α mRNA-producing cells (arrow) locate around chronic inflammation (arrow head) on the omentum. (x 20)

D), Hybridization signals of IL-1α mRNA are found in the cytoplasm of aggregated macrophage-like cells which are observed in early phase of granulomatous inflammatory response. (x 100)

Fig. 11: IL-1α mRNA-expressing vascular endothelial cells are found in the omentum with severe inflammatory response. (x 100)

Fig. 12: IL-1α mRNA-producing Kupffer cell can be seen in the liver in FIP

affected cats. (x 250)

Fig. 13: Infrequent positive hybridization is associated with the reticuloendothelial cell in the red pulp of spleen. (x 400)

Fig. 14: *In situ* hybridization analysis of feline IL-1α mRNA expression in the kidney of FIP affected cats.

A), There is a positive cell in the medullary. (x 100)

B), Hybridization signal can be seen in the cytoplasm of infiltrated mononuclear cell in the cortical areas. Hybridization signal can not be seen in the glomeruli. (x 100)

Fig. 15: IL-1α mRNA-expressing foamy cells are observed in the pancreas with severe granulomatous response. (x 200)

Fig. 16: *In situ* hybridization analysis of feline IL-1α mRNA expression in the digestive tract of FIP affected cats.

A), Infrequent signal is associated with the mononuclear cell in the mucosal layer of digestive tract. (x 120)

B), There are positive cells in the muscular layer. (x 25)

C), RNase treated serial section of Fig. 16B. There is no hybridization signal in the serial section treated by same procedure following RNase pretreatment. (x 25)

Fig. 17: IL-1α mRNA-expressing cell can be seen in the visceral pleura. (x 50)

Fig. 18: *In situ* hybridization analysis of feline IL-1α mRNA expression in the brain of FIP affected cats.

A), Several vascular endothelial cells contain IL-1α mRNA. (x 100)

B), There is positive cell on the ependyma. (x 120)

C), IL-1α mRNA is found in the ependymocytes (arrows). (x 40)

Fig. 19: IL-1α mRNA-expressing cells are localized in the epithelium layer at palpebral conjunctiva of FIP affected cats. (x 25)

Fig. 20: IL-1α mRNA-containing mononuclear cell can be seen in the bone marrow of FIP affected cats. (x 160)

TABLE

Table 1

Treatment	Poly-l-lysine (%)			
	0	0.001	0.01	0.1
Untreated	–	+	+	++
Heating				
at 37°C for 6 hours	–	+	+	++
at 37°C for 12 hours	±	+	+	++
at 37°C for 24 hours	±	+	+	++
at 60°C for 6 hours	+	+	++	++
at 60°C for 12 hours	+	+	++	++
at 60°C for 24 hours	+	+	++	++

– = No sections remained on slide throughout the ISH procedure

± = Less than 70% sections on slide throughout the ISH procedure

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

++ = 100% sections remained on slide through the ISH procedure

Table 2

Time	Proteinase K (μg/ml)				
	1	5	10	50	100
10 minutes	±	±	±	+	+
20 minutes	±	±	±	+	+
30 minutes	±	±	+	+	+

± = Weak or inconsistent reaction

+ = Moderate to strong reaction

Table 3

Time	40	50	60	70	80	90 (°C)
5 minutes	—	—	±	+	+	+
10 minutes	—	—	±	+	+	+
15 minutes	—	—	±	+	+	+

— = Negative reaction

± = Weak or inconsistent reaction

+ = Moderate to strong reaction

Table 4

Time	4	25	37 (°C)
1 hour	–	–	–
3 hours	–	±	±
5 hours	±	±	+
18 hours	±	+	+
24 hours	+	+	+

– = Negative reaction

± = Weak or inconsistent reaction

+ = Moderate to strong reaction

Table 5

Tissues	Signals	IL-1α mRNA-expressing cells
Brain	+	Vascular endothelium
Pituitary	+	Anterior pituitary cell
Eye	N.D.	
Thyroid	−	
Pleura and lung	−	
Heart	−	
Liver	+	Kupffer cells
Spleen	+	Reticuloendothelial cells and macrophages in the red pulp
Lymph nodes	+	Reticuloendothelial cells and macrophages in the sinuses
Pancreas	−	
Kidney	−	
Adrenal	−	
Uterus	−	
Digestive tract	−	
Omentum	−	
Visceral peritoneum (Serosal surface)	−	
Bone marrow	−	

+ = Detectable, − = Not detectable, N.D. = Not done

Table 6

Year when autopsied	Number of examined	Number of detected cases	Detected cases per cases examined (%)
1980	7	5	83
1981	9	7	78
1982	10	10	100
1983	3	3	100
1984	9	9	100
1985	3	3	100
1986	2	2	100
1987	2	2	100
1988	2	2	100
1989	3	3	100

Table 7

Tissues	Signals	IL-1α mRNA-expressing cells
Brain	+	Vascular endothelium, Mononuclear cells on the ependyma, Ependymocytes
Pituitary	—	
Eye	+	Epithelial cells of palpebral conjunctiva
Thyroid	—	
Pleura and lung	+	Infiltrated macrophage-like cells
Heart	—	
Liver	+	Kupffer cells
Spleen	+	Reticuloendothelial cells and macrophages in the red pulp
Lymph nodes	+	Reticuloendothelial cells and macrophages in the sinuses
Pancreas	+	Infiltrated macrophage-like cells
Kidney	+	Infiltrated macrophage-like cells
Adrenal	—	
Uterus	—	
Digestive tract	+	Macrophage-like cells
Omentum	+	Infiltrated macrophage-like cells Vascular endothelium
Visceral peritoneum (Serosal surface)	+	Infiltrated macrophage-like cells Vascular endothelium
Bone marrow	+	Mononuclear cells

+ = Detectable, — = Not detectable

FIGURE

Fig. 1

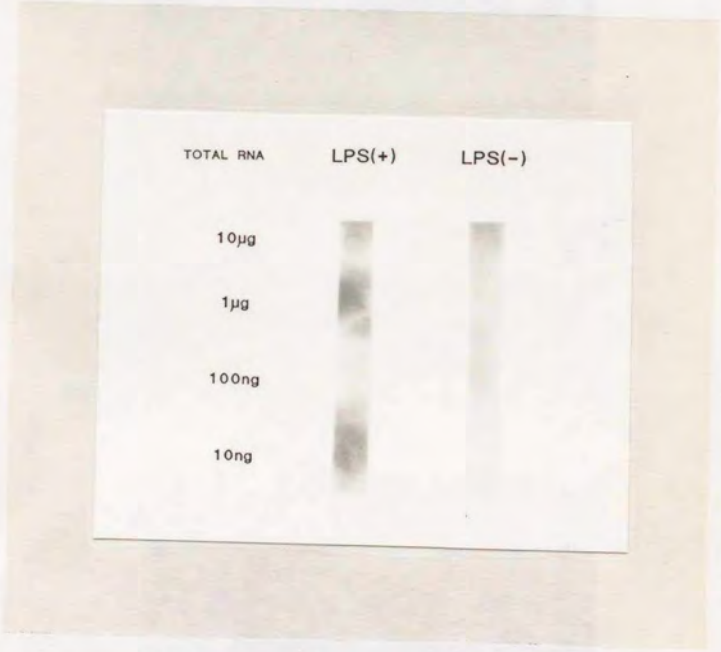
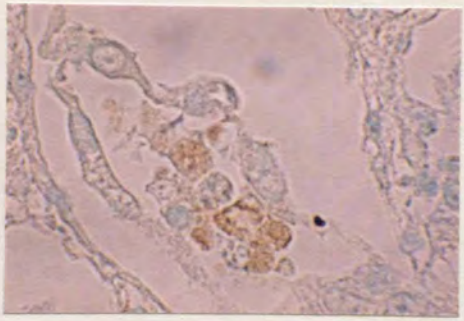


Fig. 2A

Fig. 2B



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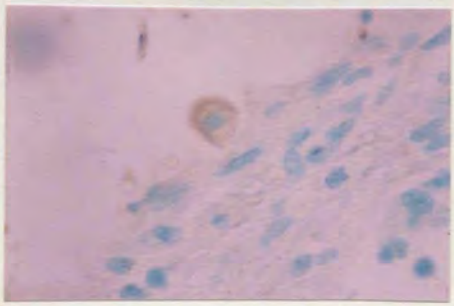
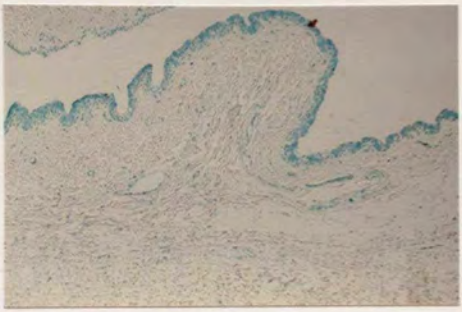


Fig. 4

Fig. 5



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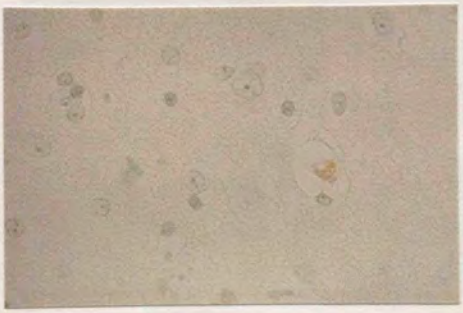
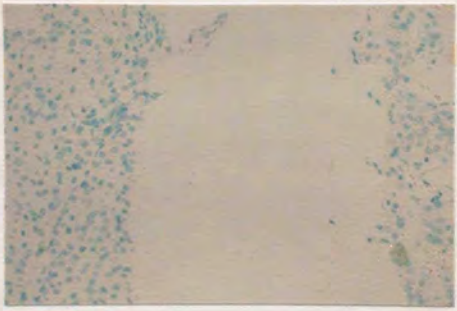


Fig. 6B

Fig. 6C



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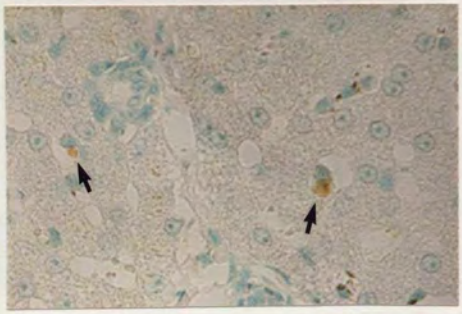


Fig. 8A

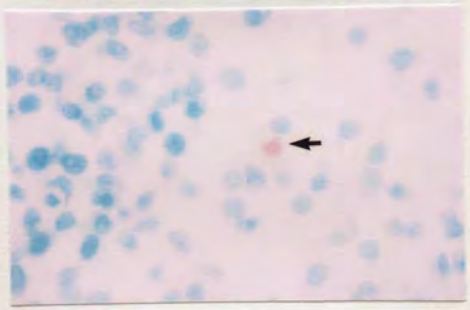
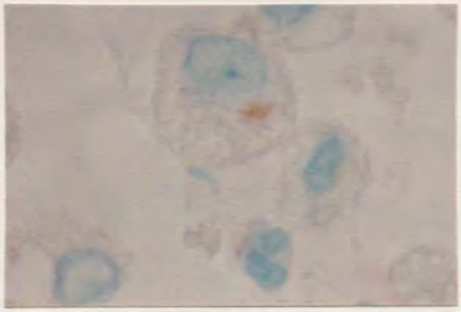


Fig. 8B



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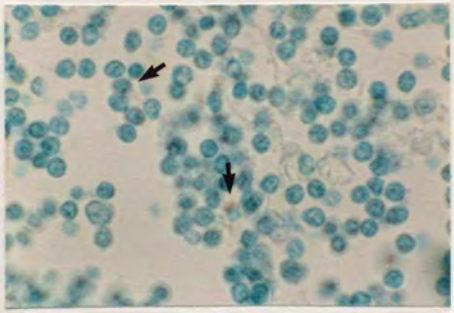
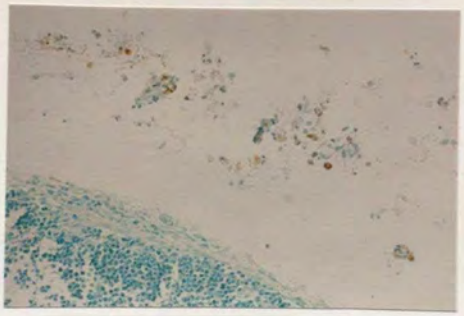


Fig. 10A



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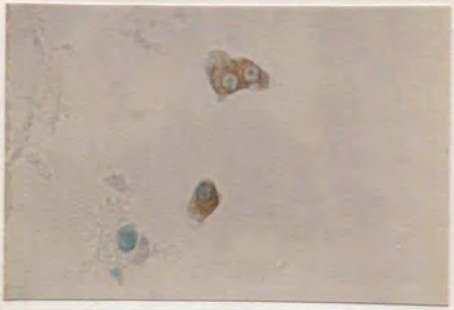
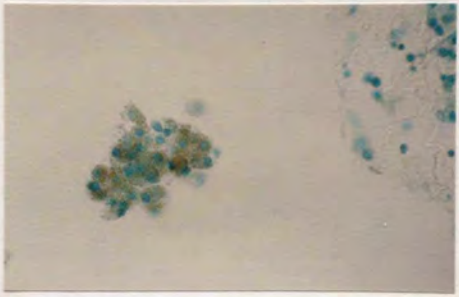


Fig. 10C

Fig. 10D



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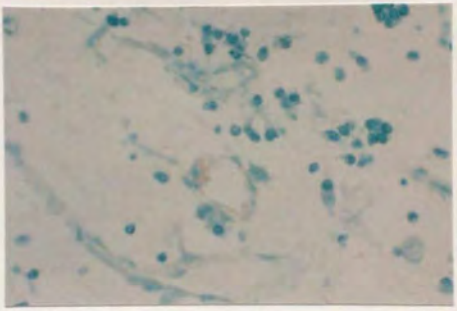

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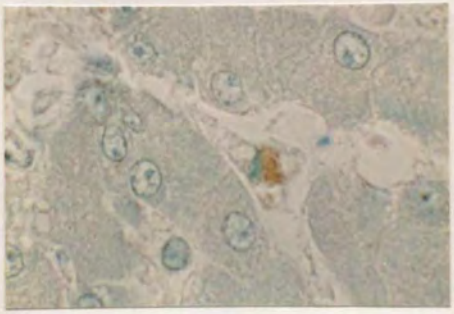

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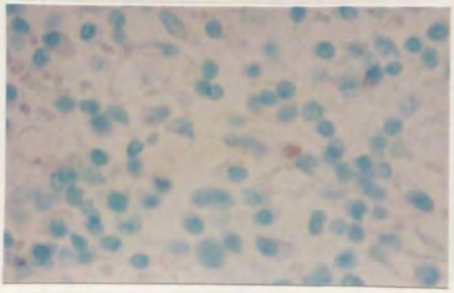

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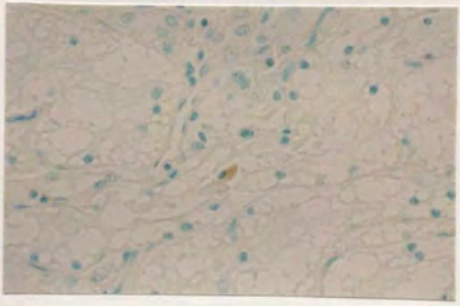


Fig. 14B

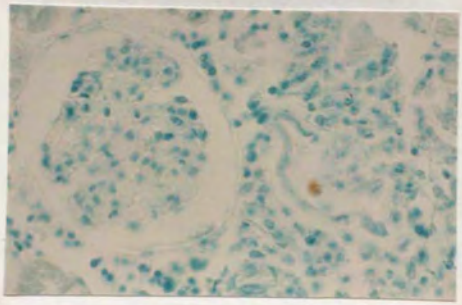


Fig. 15

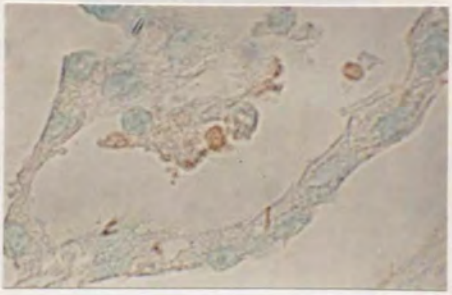


Fig. 16A

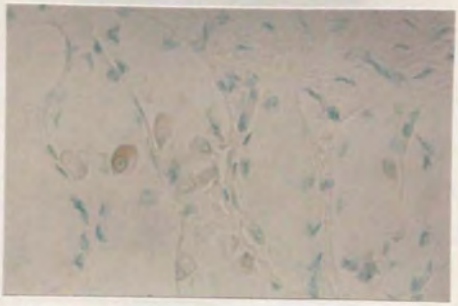


Fig. 16B

Fig. 16C

Fig. 17

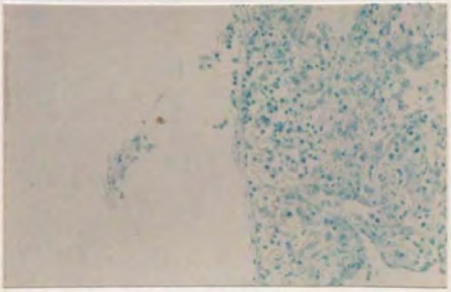
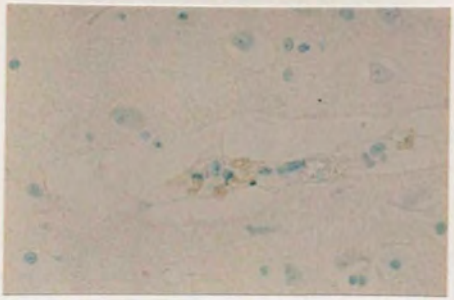


Fig. 18A



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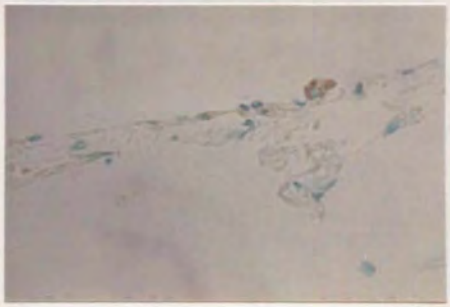
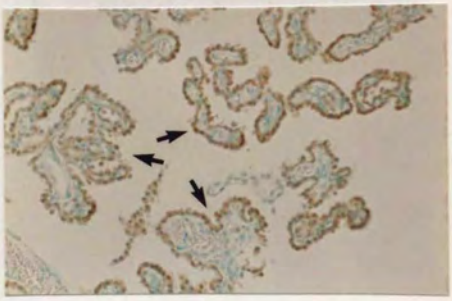


Fig. 18C



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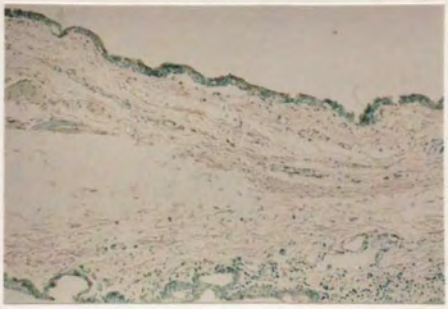

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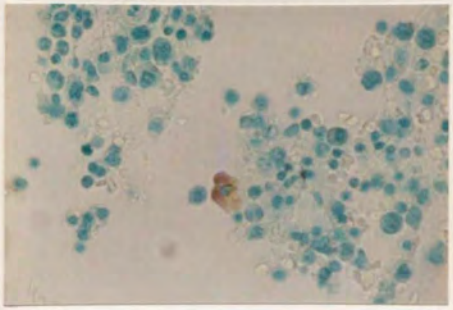

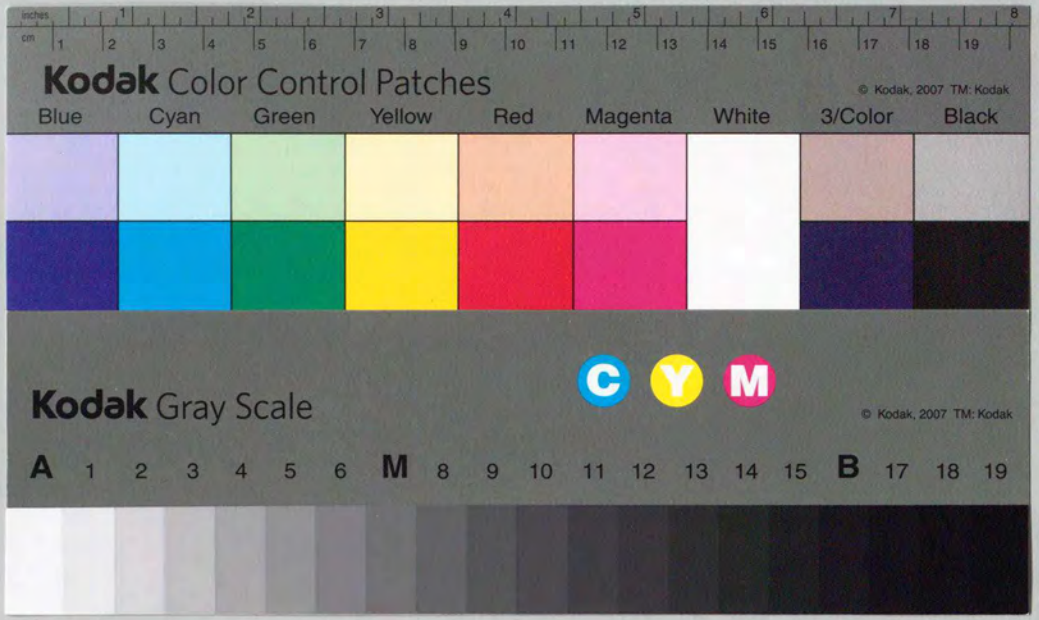
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