Histol Histopathol (2012) 27: 69-78
DOI: 10.14670/HH-27.69

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Examination of epithelial tissue cytokine response to natural peste des petits ruminants virus (PPRV) infection in sheep and goats by immunohistochemistry

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Summary. In this study, we aimed to evaluate expression of IL-4, IL-10, TNF-α, IFN-γ and iNOS in lingual, buccal mucosa and lung epithelial tissue using immunoperoxidase technique and to compare with the tissues of control animals. The tissues used in the study were collected from 17 PPRV-affected and 5 healthy sheep and goats. In PPRV positive animals, the lungs, lingual and buccal mucosa had significantly higher iNOS, IFN-γ and TNF-α expressions compared to control group animals. There was no significant difference between PPRV positive and control groups for IL-4 and IL-10 expressions of epithelial tissues. In conclusion, the epithelial tissues infected by PPRV showed significant iNOS, IFN-γ and TNF-α expressions and they might play an important role in the initiation and regulation of cytokine response, as they take place in the first host barrier to be in contact with PPRV. It is suggested that the more epithelial damage produced by PPRV the more cytokine response may result in the infected epithelial cells. The first demonstration of iNOS expression and epithelial cytokine response to PPRV in natural cases is important because it may contribute to an early initiation of systemic immunity against PPRV infection, in addition to direct elimination of the virus during the initial epithelial phase of the infection.

Key words: Epithelia, iNOS, Interferon gamma, IL4, IL10, Immunohistochemistry, Peste des Petits Ruminants, Tumor necrosis factor

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Introduction

Peste des Petits Ruminants (PPR) is either an acute or subacute viral disease of sheep and goats which can cause huge economic loss due to its high mortality and morbidity rates, associated with erosive-ulcerative stomatitis, rhinitis, pneumonia and diarrhea (Furley et al., 1987; Diallo, 1988; Scott, 1990; Barker et al., 1993; Dungworth, 1993; Alcigir et al., 1996; Dhar et al., 2002; Toplu, 2004; Kul et al., 2007, 2008).

The PPR virus (PPRV) is a member of the Morbilliviruses, which belong to the family of *Paramyxoviridae*, and has a close relationship with Rinderpest virus (RPV), canine distemper virus (CDV), measles virus (MV), dolphin distemper virus (DMV), phocine and porpoise distemper virus (PDV) (Gibbs et al., 1979; Barrett et al., 1995; Murphy et al., 1995; Barrett, 1999). PPR virus exhibits epitheliotropic features similar to other morbilliviruses. Thus, the most prominent lesions are seen in epithelial tissues of affected animals (Scott, 1981; Abraham, 2005). Syncytial cells in the oral mucosa and in proliferative lung lesions, eosinophilic intracytoplasmic and intranuclear inclusion bodies in the respiratory and/or alimentary system are considered to be the characteristic diagnostic criteria of PPRV infection. Neither systemic nor local epithelial cytokine response to PPRV infection has been documented before. Furthermore, there is no information about active cytokine and inducible nitric oxide synthase (iNOS) production in the affected epithelial tissues and syncytial cells in PPRV infection. The aims of the present study are to investigate whether iNOS expression and cytokine response (IFN-γ, TNF-α, IL-4, IL-10) to PPRV infection take place in the affected epithelia of oral mucosa and lungs, and to compare whether there is a difference between lesion severity and

the degree of epithelial cytokine and iNOS up-regulation. In this study, it is also aimed to demonstrate tissue distribution patterns of the mentioned cytokines and iNOS between control and naturally PPRV-affected sheep and goats.

Materials and methods

Animals and tissue samples

The materials of the study were paraffin embedded tissue blocks of buccal mucosa, tongue and lungs of PPR positive sheep (11) and goats (6) archived in the Department of Pathology, Faculty of Veterinary Medicine, Kirikkale University. According to their diagnostic data, each PPR infection included in the study was previously confirmed by immunoperoxidase examination and/or RT-PCR. Epidemiological, pathological and PCR findings related to some of these natural PPR cases were previously reported (Kul et al., 2007). Corresponding tissues of healthy slaughtered sheep (3) and goats (2) were also collected, fixed in 10% buffered formalin and embedded in paraffin. They were used as negative control for comparison.

Histopathology

Serial sections of each tissue were cut at a thickness of 4-5 μm, mounted on glass slides and stained with hematoxylin and eosin (HE) for histopathologic examination. A modified semiquantitative histopathologic scoring to evaluate lesion severity was used according to Moussalem et al. (2007) and one section of each tissue was evaluated under x20 microscope field. A score point was assigned for each of the following lesions; Hydropic and vacuolar degeneration, hyperkeratosis, syncitial cell formation, epithelial necrosis, pseudomembrane formation, intracytoplasmic and intranuclear inclusion bodies, inflammatory cell infiltration in epithelial layer, epithelial hyperplasia of bronchi and bronchioles, lymphoid cuffing around the airways, Type II alveolar epithelial hyperplasia, interstitial mononuclear cell infiltration, secondary bacterial infections and necrosis, and the total score was evaluated as; 1-3 points: mild, 4-5 points: moderate and 6-7 points: severe (Table 1).

The gram staining kit for demonstration of bacterial existence in the PPR lesions (BTR, Gram staining kit, Lot No:02, Kimsan Kimyevi Maddeler, Ankara, Turkey) was used.

Polyclonal anti-PPRV antibody production

To obtain polyclonal hyperimmun sera, 0,1 ml 10^3 $TCID_{50}$/ml titer PPRV Tu00 isolate was applied subcutaneously to the left plantar region of a New Zealand rabbit for five weeks at weekly intervals. Then, the rabbit was exsanguinated and its blood was collected into a silicon-coated tube. Virus neutralization test was performed to obtain hyperimmun sera and anti-PPRV polyclonal antibody. Immunoperoxidase test titer was found to be 1/16.

Immunoperoxidase examinations

A commercial streptavidin/biotin immunoperoxidase kit (HRP, Novacastra, Newcastle UK) was used to demonstrate PPRV, IL-4, IL-10, TNF-α, IFN-γ and iNOS antigens and commercial antibodies were used for the investigation of cytokine and iNOS expressions (Table 2). Deparaffinized sections were processed according to avidin biotin complex, streptavidin peroxidase technique and all steps were carried out using the commercial kit's protocol. Briefly, endogenous peroxidase activity was quenched by incubating the slides with 3% hydrogen peroxide (H_2O_2) in absolute methanol for 15 minutes. The tissues were digested with 0.1% protease K for 10 minutes at 37°C. Sections were rinsed with phosphate-buffered saline (pH 7.4) twice for 10 minutes between the consecutive steps of the test. Non-specific binding was blocked with 2% normal goat serum for 5 minutes. Tissue sections were then incubated with a primary antibody (PPRV, IL-4, IL-10, TNF-α and IFN-γ) at a dilution of 1:100 for 50 minutes in a humidity chamber. Sections were incubated with anti-mouse, anti-rat, anti-rabbit IgG secondary antibody (Novacastra Laboratories Ltd., Newcastle UK) for 15 minutes and with the streptavidin-peroxidase enzyme for 15 minutes at room temperature, and finally sections were incubated in aminoethyl carbasole (AEC) chromogen (Zymed Laboratories Inc., USA) for 5-10 minutes, counterstained with Mayer's hematoxylin for 1-2 minutes, dehydrated, and mounted with aqueous mounting medium. Sections investigated for iNOS expression were incubated with 3,3'-diaminobenzidine (DAB) chromogen (Neomarkers, Fremont CA) for 5-10 min, counterstained very lightly with Mayer's haematoxylin for 1-2 min, dehydrated, and mounted with mounting medium. For staining control, PBS was used instead of primary antibody for immunhistochemistry of each tissue (Inserted in the upper right of the Figs. 1D,I,J, 2D,I).

Double labelling immunohistochemistry for iNOS + TNF-α and iNOS + IFN-γ

Commercial rabbit anti-bovine IFN-γ and TNF-α antibodies (Table 2) were used to detect IFN-γ and TNF-α antigens. To demonstrate iNOS, mouse anti-iNOS antibody (Neomarkers, Fremont CA) was used. Double labelling immunohistochemistry was performed to demonstrate co-localisation of iNOS + IFN-γ and iNOS + TNF-α antigens. A HRP kit (Dako, Glostrup, Denmark) was used for iNOS antigen and an Alkaline Phosphatase kit (Labvision corporation, Fremont CA) was used for IFN-γ and TNF-α antigens. The sections were digested with 0.1 % proteinase K (VMRD, Inc., USA) for 10 min and blocked with a protein blocking

agent (Labvision Corp., Fremont, CA) for 5 min. The sections were then incubated with mouse anti-iNOS antibody for 50 min. Following application of an anti-mouse biotinylated polyvalent secondary antibody for 10 min, strepavidin conjugated with horse radish peroxidase enzyme was applied for 10 min and 3,3'-Diaminobenzidine (DAB) chromogen substrate solution was incubated for 10-20 min for colour reaction. The sections were rinsed with distilled water. Then, rabbit anti-IFN-γ or TNF-α antibody (1:100 diluted) was applied onto sections for 50 min, at room temperature. Following application of an anti-rabbit biotinylated polyvalent secondary antibody for 10 min and then streptavidin alkaline phosphatase enzyme for 10 min, 5-Bromo-4-chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) (Thermoscientific, USA) was applied onto sections for 30 min for colour reaction. The cytoplasms were stained blue/purple in cytokine-positive cells and brown in iNOS positive cells. Dark brown and co-associated blue color demonstration in a cell was evaluated as co-localisation of iNOS+TNF-α or iNOS+IFN-γ antigens. No counter staining was used in double immunohistochemistry.

Histomorphometric analysis and statistics

Mann-Whitney U-test, on the evaluation of non-parametric data, was used to compare IL-4, IL-10, TNF-α and IFN-γ immunoreactive cells and immuno-positively stained areas in animals with PPR and healthy controls. A p value less than 0.05 was considered statistically significant. In the evaluation of intracellular and interstitial immunostainings, Bs200ProP (BAB image analysis system, Turkey) was used. Briefly, at least 3 randomly selected and consecutive 40x objective microscope fields were photographed. After calculation of proportional (% pixels) staining area to whole fields, the mean % pixels staining area for each case was calculated.

Staining results for iNOS, each individual sample was graded using the following reaction score: (no expression, -); (faint expression, +); (moderate

Table 1. Histopathological lesions and scores of each control and PPRV-affected animal. Total histopathologic scores, Gram staining and anti-PPRV immunoperoxidase findings of tongue, buccal mucosa and lung tissues of all animals included in the study.

Lesions/Case n°	Buccal mucosa (BM) / Tongue epithelium (TE)												Lung									
	Species	Age	HVD	HP	SHF	EN	PO	ISC	HI	BM/TE Total lesion score	Gram Staining	Immuoperoxidase	BH	PL	F	AL	IMH	IC	SBP	Lung Total lesion score	Gram Staining	Immuoperoxidase
Contol group animals																						
1	S	2y	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-	-/-	0	0	0	0	0	0	0	0	-	-
2	S	2y	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-	-/-	0	0	0	0	0	0	0	0	-	-
3	S	2y	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-	-/-	0	0	0	0	0	0	0	0	-	-
4	G	2y	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-	-/-	0	0	0	0	0	0	0	0	-	-
5	G	2y	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-	-/-	0	0	0	0	0	0	0	0	-	-
PPRV positive animals																						
6	S	1y	1/1	1/0	1/0	1/1	1/0	1/1	1/0	7/3	p/p	+/+	0	0	1	1	1	1	1	5	p	+
7	S	4m	1/1	1/0	1/0	1/0	0/0	1/0	1/0	6/1	p/p	+/-	0	0	1	1	1	1	1	5	-	+
8	S	3m	1/0	1/1	1/1	1/1	1/1	1/1	1/1	7/6	p/-	+/+	1	0	0	0	1	0	1	3	-	-
9	S	4m	1/1	0/0	0/0	0/0	0/0	0/0	1/0	2/1	-/-	-/-	0	0	1	0	1	0	0	2	-	+
10	G	3y	1/0	1/1	0/1	1/1	1/1	0/1	1/1	5/6	-/-	+/+	1	1	1	1	1	1	1	7	p	+
11	G	4y	1/1	1/0	1/0	1/0	1/0	1/0	1/0	7/1	-/p	+/-	1	1	1	1	1	0	0	5	-	+
12	G	3y	1/0	1/1	1/1	1/1	1/1	1/1	1/1	7/6	p/p	+/+	1	1	1	1	1	1	1	7	p	-
13	S	2m	1/0	1/1	1/1	1/1	1/1	1/1	1/1	7/6	p/-	+/+	1	0	1	1	0	1	1	5	-	+
14	S	3y	1/0	0/1	0/0	0/0	0/0	0/0	0/0	1/1	-/-	-/-	0	0	1	1	0	1	1	4	-	+
15	S	3y	1/0	0/1	0/0	0/1	0/0	0/0	0/0	1/2	-/-	-/+	0	1	0	1	0	0	0	2	-	+
16	S	3y	1/0	1/1	0/0	0/0	0/1	0/0	0/0	2/2	-/-	+/+	1	0	1	0	1	0	0	3	-	-
17	S	3y	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	-/-	-/-	0	0	1	1	0	0	1	3	-	+
18	G	1m	1/0	0/1	0/0	0/0	0/0	0/0	0/0	1/1	-/-	+/-	0	0	1	1	0	1	0	3	-	+
19	S	3m	1/0	0/1	0/0	0/0	0/0	0/0	0/0	1/1	-/-	-/-	1	0	1	1	0	1	0	4	-	+
20	G	3m	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	-/-	-/+	1	1	0	0	0	0	0	2	p	+
21	S	2y	1/0	0/1	0/0	0/0	0/0	0/0	0/0	1/1	-/-	-/-	0	0	1	1	0	0	0	2	-	+
22	G	3m	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	-/-	-/-	1	1	0	0	0	0	0	2	p	+

HVD: hydropic, vacuolar degeneration; HP: Hyperkeratosis; SHF: syncitial cell formation; EN: epithelial necrosis; PO: pseudomembranes formation; ISC: intracytoplasmic and intranuclear inclusion bodies; HI: inflammatory cell infiltration in epithelial layer; BH: bronchi and bronchioles epithelial hyperplasia; PL: peribronchial, peribronchialar lymphoid cuffing; F: Type II alveolar epithelial hyperplasia; AL: syncitial cell formation in alveoli; IMH: interstitial mononuclear cell infiltration; IC: inclusion bodies; SBP: secondary bacterial infections and necrosis; BM/TE: Buccal mucosa / Tongue epithelium; G: goat; S: sheep; 0: no lesion; 1: lesion existing; p: gram positive; -: No staining; +: positive staining.

expression, ++); (strong expression, +++); (very strong expression, ++++).

Results

Histopathologic findings

Histopathologic findings and overall lesion scores of the examined tissues are summarized in Table 1. Hydropic and vacuolar degeneration in the epithelium of lingual (Fig. 1A) and buccal mucosa were the consistent findings in PPRV-affected animals. Hyperkeratosis, necrotic and pseudomembraneous stomatitis in buccal (case Nos. 6, 8, 10-13, 16) and lingual mucosa (case Nos. 8, 10, 12-16, 18, 19, 21) were seen. Syncytial cells were observed in the degenerative and necrotic epithelium of stratum spinosum in buccal mucosa (case Nos. 6-8, 11-13) and lingual mucosa (case Nos. 8, 10, 12, 13). Intracytoplasmic and intranuclear eosinophilic inclusion bodies were co-associated with these lesions (Fig. 1A).

Acute proliferative interstitial pneumonia characterized by peribronchial and/or peribronchiolar cuffing (case Nos. 10-12, 15, 20, 22) and thickening in interalveolar septa (case Nos. 6-12, 16) were observed in the lungs of animals with PPR. Type II alveolar epithelium hyperplasia (foetalisation) was detected in 13 out of 17 cases (case Nos. 6, 7, 9-14, 16-19, 21). Syncytial cell formations (Fig. 1B) in the alveoli were present in 12 out of 17 cases (case Nos. 6, 7, 10-15, 17-19, 21). Eosinophilic viral inclusions were seen in Syncytial cells and epithelial cells of bronchi and bronchiole (case Nos. 6, 7, 10, 12-14, 18, 19).

Gram staining results

Gram staining results are given in Table 1. Clusters of Gram (+) positive bacteria were seen in 5 out of 17 buccal mucosa (case Nos. 6-8, 12, 13), in 4 out of 17 lingual mucosa (case Nos. 6, 7, 11, 12) and in 5 out of 17 lungs (case Nos. 6, 10, 12, 20, 22) (Fig. 1C).

Immunoperoxidase findings

Immunoperoxidase staining of PPRV antigen

Intense PPRV antigen immunopositive reactions were detected in the degenerative and necrotic epithelium (Fig. 1D), and in morphologically normal epithelial cells localized in the germinative epithelium of epidermis. Syncytial cells (case Nos. 8, 12, 13) in buccal mucosa and lingual epithelium was showed strong

Table 2. Commercial cytokines used in immunochemistry.

Company	Antibody	Antibody type	Host Antibody	Antigen retriavel method	Dilution in use
Thermo Scientific	IL-4	Polyclonal	Rabbit	ProteinaseK (%0.1)	1/100
Thermo Scientific	IL-10	Polyclonal	Rabbit	ProteinaseK (%0.1)	1/100
Thermo Scientific	TNF-α	Polyclonal	Rabbit	ProteinaseK (%0.1)	1/100
Thermo Scientific	IFN-γ	Polyclonal	Rabbit	ProteinaseK (%0.1)	1/100
Thermo Scientific	iNOS	Polyclonal	Mouse	ProteinaseK (%0.1)	1/100

Table 3. Cytokine immunoperoxidase test results and statistical data.

Cyokine	Tissue	Control group animals		PPRV positive animals		Statistical significance (p< 0.05)
		Mean	Std. deviation	Mean	Std. deviation	
IFN-γ	Lung	0,606	0,404	2,267	2,321	0,031*
	Buccal mucosa	0,007	0,001	2,798	2,702	0,003*
	Tongue	0,006	0,001	1,461	1,198	0,003*
TNF-α	Lung	0,030	0,0261	0,299	0,614	0,011*
	Buccal mucosa	0,001	0	0,546	0,711	0,031*
	Tongue	0,001	0	0,445	0,588	0,048*
IL-4	Lung	0,010	0,004	0,010	0,002	0,880
	Buccal mucosa	0,010	0	0,024	0,059	0,880
	Tongue	0,012	0,001	0,048	0,145	0,880
IL-10	Lung	0,011	0,002	0,011	0,002	0,820
	Buccal mucosa	0,010	0,002	0,010	0,002	0,704
	Tongue	0,010	0,004	0,015	0,006	0,120

* similarity less than 0.05 (p< 0.05) was significant.

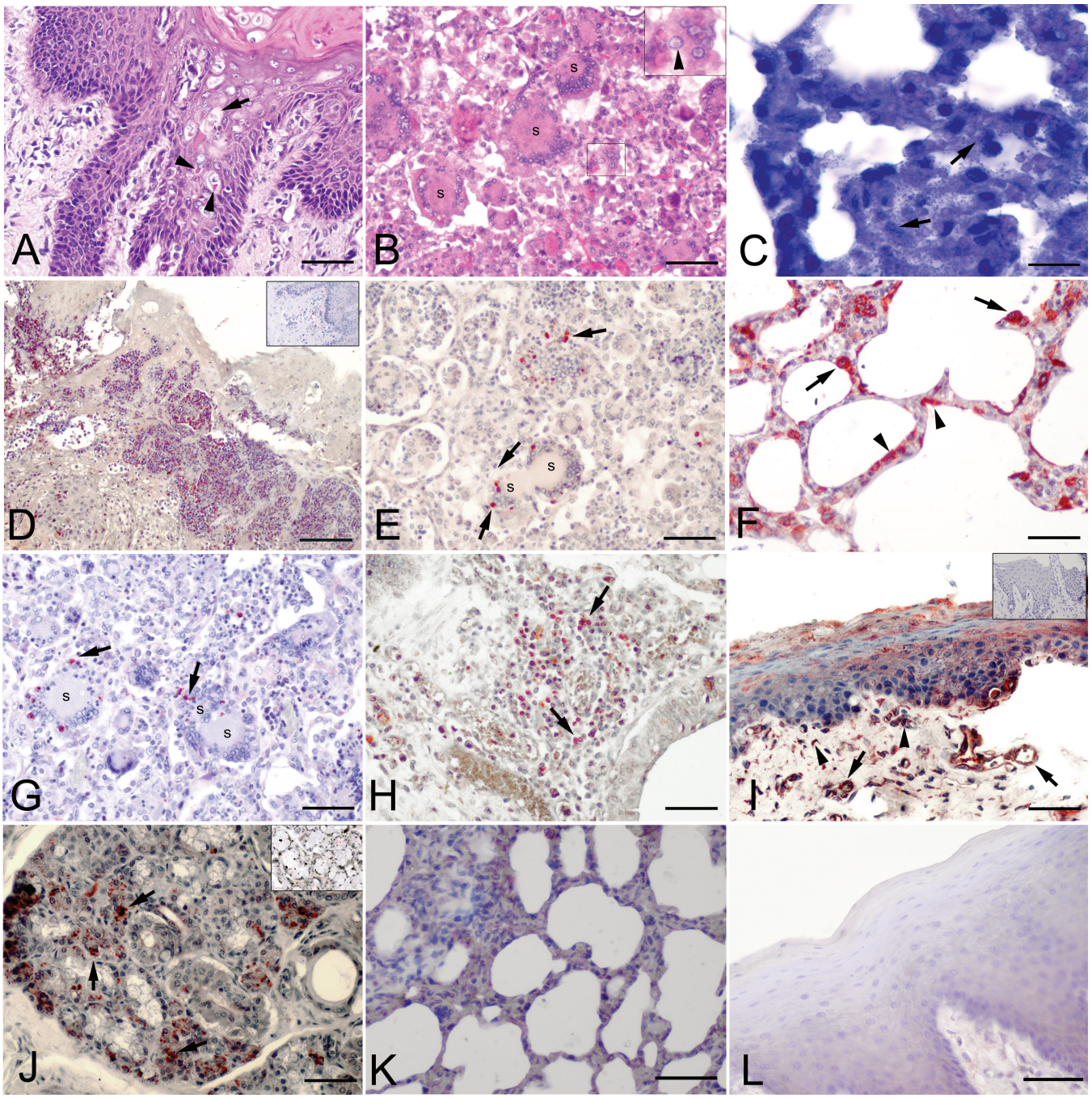

Fig. 1. A. Lingual tissue; sheep, case No.8. Hydropic and vacuolar degeneration (arrow) and intracytoplasmic inclusion bodies (arrow heads). HE. **B.** Lung; goat, case No.10. Syncytial cells (s) and intranuclear inclusion bodies. HE. **C.** Lung; sheep, case No.7. Cluster of bacteria in phagocytic cell cytoplasm (arrows) in bronchopneumonic lesion. Gram Staining. **D.** Buccal mucosa; goat, case No.11. Immunoreactivity in mucosa epithelium against PPRV antigen. ABC technique (anti-PPRV), Mayer's hematoxylin counterstain. Upper right figure demonstrates that there is no non-specific background in negative control staining slide. **E.** Lung; sheep, case No.13. Immunoreactivity in syncytial cells (s) (arrows). ABC technique (anti-PPRV), Mayer's hematoxylin counterstain. **F.** Lung; sheep, case No.9. Immunoreactivity in alveoli epithelium cell (arrow heads) and pulmonary intravascular monocytes (arrows). ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. **G.** Lung; sheep, case No.6. Immunoreactivity in syncytial cells (s) (arrows). ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. **H.** Lung; goat, case No.11. Immunoreactivity in mononuclear cells in peribronchial cuffing (arrows). ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. **I.** Lingual tissue; sheep, case No.8. Immunoreactivites in mucosal epithelium, and in submucosa, capillary vessels endothelial cell (arrows) and fibroblasts (arrow heads). ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. Upper right figure demonstrates that there is no non-specific background in negative control staining slide. **J.** Buccal mucosa; sheep, case No.6. Immunolabelling in gland epithelial cells in submucosa (arrows). ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. Upper right figure demonstrates that there is no non-specific background in negative control staining slide. **K.** Lung; sheep, control group, case No.1. No immunolabelling in alveolar epithelium and interalveolar area. ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. **L.** Buccal mucosa; sheep, control group, case No.3. No immunolabelling mucosa epithelium. ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. Bars: A, B, E-L, 50 μm; C, 20 μm; D, 200 μm;

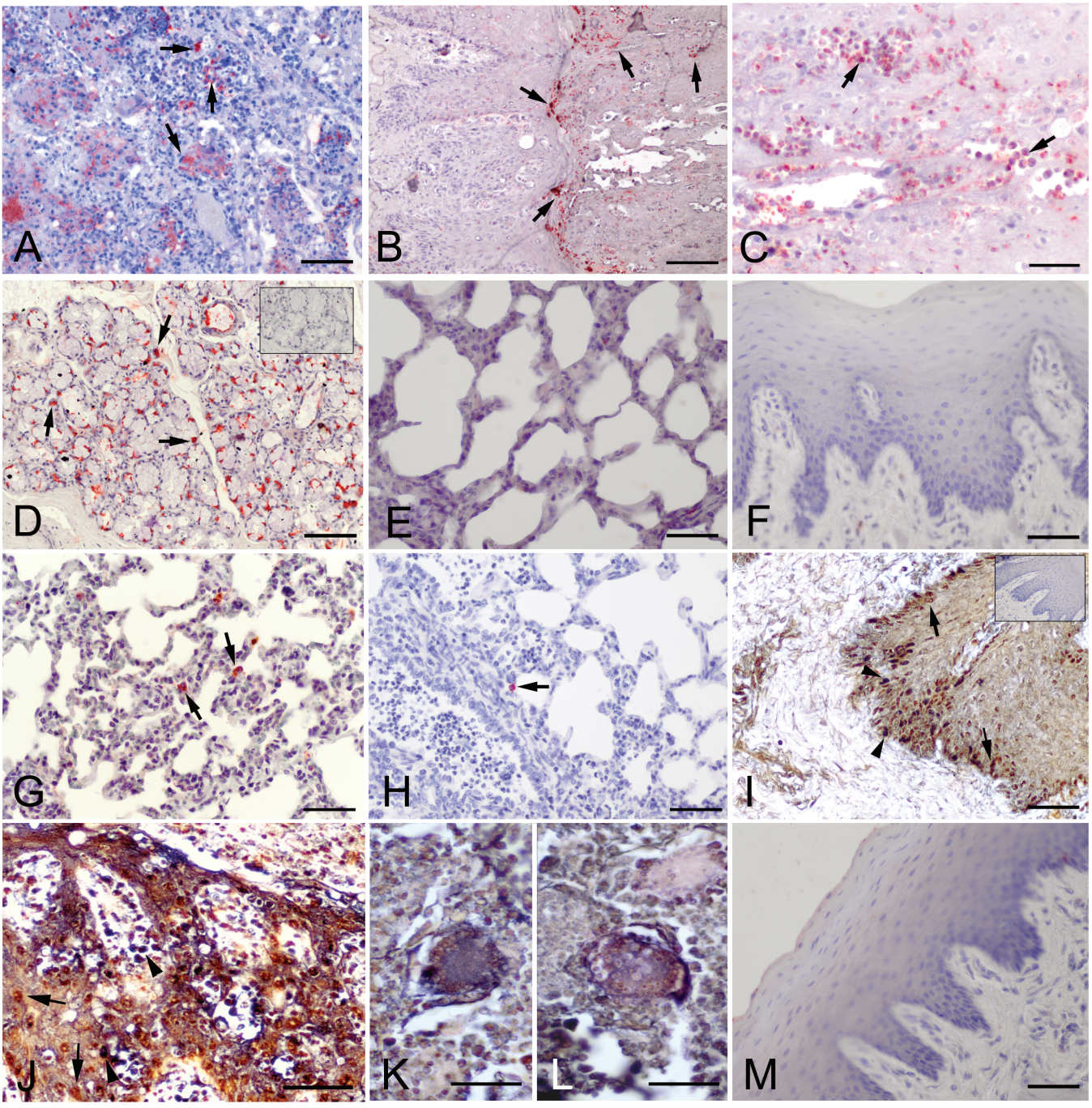

Fig. 2. A. Lung; goat, case No.10. Immunoreactivity in bronchopneumonic areas (arrows). ABC technique (anti-TNF-α), Mayer's hematoxylin counterstain. **B.** Buccal mucosa; goat, case No.12. Immunolabeling in basal cells and in pseudomembrane (arrows). ABC technique (anti-TNF-α), Mayer's hematoxylin counterstain. **C.** Buccal mucosa; sheep, case No.13. Immunoreactivity in mononuclear cells in mucosa (arrows). ABC technique (anti-TNF-α), Mayer's hematoxylin counterstain. **D.** Buccal mucosa; goat, case No.12. Immunolabelling in gland epithelial cells in submucosa (arrows). ABC technique (anti-TNF-α), Mayer's hematoxylin counterstain. Upper right figure demonstrates that there is no non-specific background in negative control staining slide. **E.** Lung; sheep, control group, case No.2. No immunolabelling in alveolar epithelium and interalveolar area. ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. **F.** Buccal mucosa; sheep, control group, case No.3. No immunolabelling mucosa epithelium and submucosa. ABC technique (anti-IFN-γ), Mayer's hematoxylin counterstain. **G.** Lung; goat, case No.11. Interleukin-4 positive cells in alveolar septa (arrows). ABC technique (anti-IL-4), Mayer's hematoxylin counterstain. **H.** Lung; goat, case No.11. Immunoreactivity in mononuclear cells in interalveaolaris (arrow). ABC technique (anti-IL-10), Mayer's hematoxylin counterstain. **I.** Buccal mucosa; goat, case No.12. iNOS was absent from the greater part of basal layer and gained intensity in upper basal epithelial cell layers. iNOS+TNF-α co-localizations were evidenced. Brown stained cells positive for iNOS (arrows). Brown with blue stained cells were iNOS+TNF-α positive cells. Double immunolabelling method. No counterstain was used. Upper right figure demonstrates that there is no non-specific background in negative control staining slide. **J.** Buccal mucosa; sheep, case No.6. Staining for iNOS (arrows) was represented by a cytoplasmic reaction. iNOS+IFN-γ colocalizations (arrow heads) were seen in epithelium. Double immunolabelling method. No counterstain was used. **K.** Lung; goat, case No.10. Syncytial cells stained positive for iNOS+TNF-α. Double immunolabelling method. No counterstain was used. **L.** Lung; goat, case No.12. Syncytial cells stained positive for iNOS+IFN-γ. Double immunolabelling method. No counterstain was used. **M.** Buccal mucosa; control group, case No.2. There were no examples of iNOS immunostaining in the epidermal cells. ABC technique (anti-iNOS), Mayer's hematoxylin counterstain. Bars: A, 100 μm; B, D-M, 50 μm; C, 25 μm;

immunoreactivity.

Intense PPRV immunoreactivity was observed in alveolar epithelium, neutrophil leukocytes and in alveolar macrophages in the alveolar lumens (case Nos. 6, 7, 10, 13-14, 17). Alveolar syncytial cells (Fig. 1E) were immunopositively stained both in intranuclear and intracytoplasmic localization (case Nos. 6, 7, 10, 13, 17, and 19). Alveolar macrophages, epithelium of bronchi and bronchiole (case Nos. 10, 11, 13-15) and interstitial mononuclear cells were PPRV antigen immunoreactive as well.

Cytokine expression in the epithelial tissues

Statistical evaluation of IFN-γ, TNF-α, IL-4 and IL-10 antigen expressions in the tissues of PPRV-affected and control animals are given in Table 3.

IFN-γ expression

Epithelial cells lining the oral cavity, mostly degenerated and normal epithelial cells in Stratum spinosum, exhibited strong IFN-γ expression (Fig. 1I). Necrotic debris and hyperkeratotic material overlying the pseudomembraneous oral mucosa also contained intense granular immunostainings. In the submucosa, capillary endothelia, fibroblasts and nuclei of myocytes had IFN-γ immunolabelling. Seromucous salivary gland epithelium (Fig. 1J) in the submucosa was also noticed as immunopositive. In control tissues, a few lymphocytes in interalveolar septum were immunopositive, and oral cavity epithelium showed a mild immunopositivity.

In the present study, the percentage of areas showing IFN-γ positive staining in the lungs, lingual, and buccal mucosa of PPR-positive animals was significantly higher when compared with that of the control ($p<0.05$). The highest mean value for positively stained areas was observed in the buccal mucosa. Positively stained areas were observed in the lungs (bronchial and bronchiolar epithelial cells, Syncytial cells), and lingual and buccal mucosa (stratum spinosum epithelial cells, submucosal capillary endothelium, and fibrocytes). In the control group, IFN-γ immunopositive cells were markedy few, and there was statistically significant difference between the PPRV-positive animals and the control group.

IFN-γ antigen immunoreactivity was also seen in neutrophil leucocytes, and suggestive of intravascular monocytes (Fig. 1F) more specific to bronchopneumonia consistent lesions in the lungs of PPRV-affected animals (case Nos. 6-8, 10, 12-14, 17). Syncytial cells (Fig. 1G) and mononuclear cells localized in interalveolar septa, peribronchial lymphoid cells (Fig. 2B) and bronchial epithelium showed cytoplasmic immunopositivities. Epithelial cells lining the oral cavity, mostly degenerated and normal epithelial cells in Stratum spinosum, exhibited strong IFN-γ expression (Fig. 1H). Necrotic debris and hyperkeratotic material overlying the pseudomembraneous oral mucosa also contained intense granular immunostainings. In the submucosa, in capillary endothelia, fibroblasts (Fig. 1I) and nuclei of myocytes were shown positive IFN-γ immunolabelling. Seromucous salivary gland epithelium (Fig. 1J) in the submucosa was also noticed as immunopositive. There was no significant IFN-γ immunoreactivity in control animal (Fig. 1K,L) tissues, except for a few interstitial cells in the lungs and a faint immunopositivity in the submucosal cells in oral cavity mucosa.

TNF-α expression

TNF-α immunoreactivity in the lungs of PPRV positive animals was observed in bronchopneumonic lesions (Fig. 2A) and in necrotic areas, in addition to interstitial lymphocytes, Syncitial cells and alveolar

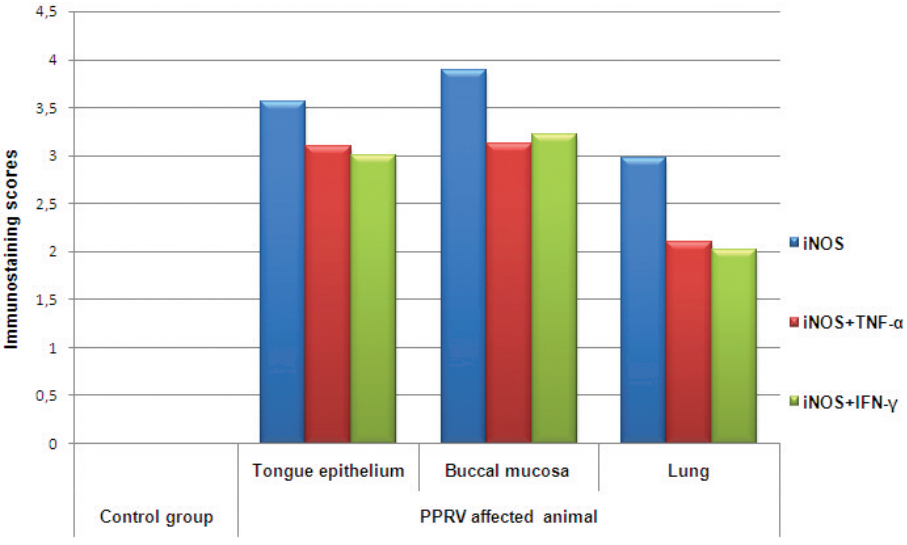

Fig. 3. Semiquantitative analysis of iNOS, iNOS+TNFα and iNOS and IFN-γ mean) in PPRV affected tissues and uninfected control animals. iNOS, iNOS+TNF-α, iNOS+IFN-γ results were not evaluated statistically and a semiquantitative scoring was performed because the control group showed no immunostaining (value was "0").

macrophages. TNF-α immunopositivity was high among the necrotic epithelial cells and pseudomembrane formation (Fig. 2B). TNF-α expression was seen not only mononuclear cells in mucosa (Fig. 2C), but also in submucosa. Salivary gland epithelial cells (Fig. 2D) and fibroblasts in submucosa were also noticed as immunopositive. There was no remarkable TNF-α (Fig. 2E,F) immunoreactivity in control animal tissues except for a few interstitial cells in the lungs and a mild immunopositivity in the submucosal cells in oral cavity mucosa.

In the control group, TNF-α immunopositive cells were markedly few, and there was a statistically significant difference between the PPRV-affected animals and the control group.

IL-4 and IL-10 expression

IL-4 and IL-10 positive cells were observed around the vessels, bronchi, bronchioles, and in interalveolar septum (Fig. 2G,H) in PPRV-affected animal tissues. Fibroblasts under basement membrane were immunoreactive in buccal mucosa and lingual epithelium. There was no statistically significant difference between the PPRV-affected animals and the control group.

iNOS expression and iNOS + TNF-a iNOS + IFN-g co-localisation in the epithelial cells

iNOS were expressed by dermal macrophages in all cases. Staining for iNOS was represented by a cytoplasmic reaction and occasionally included a strong, almost homogeneous staining of the whole nucleus. Epithelial cells in buccal mucosa and fibrocytes in submucosa and syncytial cells (Fig. 2K,L), as well as intravascular monocytes, stained positive for iNOS, iNOS+TNF-α and iNOS+IFN-γ. iNOS was absent from the greater part of basal layer and gained intensity in upper basal epithelial cell layers. Similarly iNOS+TNF-α and iNOS+IFN-γ co-localizations were evidenced in most of these cells (Fig. 2I,J). In control animals, there were no examples of iNOS, iNOS+TNF-α and iNOS+IFN-γ immunostaining in the epidermal cells (Fig. 2M) and lungs. In Fig. 3, immunolabelling of iNOS and double labeling immunohistochemistry in PPRV affected and control group animal tissues is demonstrated.

Discussion

Local expression of cytokines and iNOS can be detected by immunohistochemistry in formalin fixed paraffin embedded tissues (Kipar et al., 2003; Thanawongnuwech et al., 2003; Lorenzo et al., 2006; Moussallem et al., 2007; Solanki et al., 2009; Vilani-Moreno et al., 2011). The degree of PPRV positive staining cells in the erosive and ulcerative areas and germinative epithelial cells were found to be similar immunostaining to the iNOS, IFN-γ and TNF-α expression. Releasing of iNOS, TNF-α and IFN-γ in those areas may lead to the conclusion that epithelial tissue affected by the PPRV can directly contribute to the immune response.

Kock et al. (1990) reported that following ultraviolet irradiation human keratinocytes could produce TNF-α, which plays an important role in the pathogenesis of both local and systemic inflammatory response against microbial organisms and tumors. In the current study, TNF-α immunoreactivity in the epidermis was found to be increased in comparison with control animals. TNF-α is a proinflammatory cytokine (Dinarello, 2000; Basset et al., 2003) that plays an active role in generation of the cell-mediated immune response (Opal and Depalo, 2000). The elevated levels of TNF-α expression and co-localisation of iNOS and TNF-α in the epithelium in PPRV-affected animals are associated with viral invasion of epithelial cells. In other words, epithelial cells which are in contact with the virus may be responsible for generating the immune response required for the initiation of the inflammation.

IFN-γ positive stainings in the epithelial cells of lingual and buccal mucosa are the indicators of an active contribution to an antiviral activity. Maeyer and Maeyer-Guignard (1998) reported that IFN-γ had a direct antiviral influence and exhibited it through oligoadenylate synthetase enzyme. Moreover, IFN-γ is known to mostly demonstrate its antiviral effect with cytokines such as IFN-γ, IFN-ß and TNF-α.

It is shown for the first time that iNOS, IFN-γ and TNF-α expression in the alveolar macrophages and in syncytial cells in PPRV infection in the present study. Infection of dendritic cells with the Morbilliviruses may cause fusion between dendritic cells and/or macrophages, and formation of a syncytial cell (giant cell), rendering antigen presenting function impossible and providing a suitable environment for virus proliferation (Moussallem et al., 2007). Currently, while syncytial cells cannot be immunophenotypically proposed as having a macrophage-like characteristic, release of Th1 cytokines by them suggests that they can process the antigen and take part in its modulation. Moussallem et al. (2007) showed that the percentage of IFN-γ immunopositivity over the alveolar areas of 42 children who died of measles was higher when compared with the control group. However, they also reported that intergroup differences were not statistically significant with regard to TNF-α staining. Higher concentrations of IFN-γ and TNF-α in PPRV-affected animals observed in the present study show that PPRV and measles viruses have different pathogenesis. Svitek and Von Messling (2007) found no cytokine expression in peripheral blood leukocytes in ferrets with canine distemper virus infection. Those animals yielding to the infection failed to build up a sustained response, while survivors showed initially a Th1 polarization that later turned into a Th2-biased response. In the present study, statistically significant IFN-γ positivity, a Th1 cytokine,

was found to be consistent with the study of Svitek and Von Messling (2007). PPRV infected sheep and goats died in the peracute and acute phases that has been indicated that proinflammatory cytokine response is the first factor involved in pathogenesis of the infection and cannot turn the Th2-biased response.

iNOS expression generally occurs after cytokine response in the keratinocytes (Stallmeyer et al., 1999; Chang et al., 2003) and fibroblasts (Wang et al., 1997), as well as in Langerhans cells (Qureshi et al., 1996). Cutaneous cells and keratinocytes give the impression of being the main target for iNOS induction (Becherel et al., 1994). Proinflammatory cytokine synthesis in keratinocytes is partly under the control of the NO pathway (Becherel et al., 1994) and iNOS level may be evaluated as a cellular immune response marker and an indicator of IFN-γ production (Little et al., 2001). In the present study, we investigated whether iNOS+ keratinocytes could synthesize these cytokines. In PPRV affected animals, almost all iNOS+ lingual and buccal mucosa epithelial cells also showed TNF-α and IFN-γ expression. The most demonstrative co-localization of iNOS and TNF-α/IFN-γ were present in basal keratinocytes. The authors believe that iNOS immunostaining of epidermal cells could be the reactive Langerhans cells and/or exhibit direct contribution of iNOS production in keratinocytes.

In this study, no statistically significant difference was found between the PPRV-affected animals and the control group in terms of IL-4 and IL-10 concentrations. IL-4 is a cytokine which inhibits monocyte activation stimulated by IFN-γ (Te Velde et al., 1990), cytotoxic activity of macrophages, and proinflammatory cytokine release and expression, while suppressing the production of cytokines secreted from monocytes and nitric oxide released by macrophages (Opal and Depalo, 2000). IL-10 has been reported to suppress production of TNF-α and IL-1 from macrophages, while reducing production of reactive nitrogen intermediates and expression of nitric oxide synthesis (Laskin et al., 2007). In view of this report, high TNF-α concentration reveals the insignificant positivity of IL-10. In other words, it indicates that the level of released IL-10 was not adequate enough to influence TNF-α concentration.

In conclusion, animals can die after the Th1 immune response aimed at preventing virus proliferation. Local Th1 cytokine response in lingual, buccal mucosa, and lung tissues during PPRV infection is initiated before the occurrence of Th2 response which generates humoral reaction. The co-localization of iNOS and pro-inflammatory cytokines within keratinocytes in PPRV affected animals demonstrates that these cells play an important role in the initiation and maintenance of the inflammatory reaction during the disease. Investigation of both local and systemic cytokine responses will provide more detailed information on the understanding of the immunopathogenesis of PPR. The authors believe that demonstration of prevalent pro-inflammatory cytokine response locally in the epithelium of oral mucosa and lungs has provided information on the immunopathogenesis of erosive ulcerative stomatitis and syncytial cells which are known to be the two most important histopathological findings of PPR.

Acknowledgements. The authors wish to thank Assistant Professor Serkan ERAT from Kirikkale University, Faculty of Veterinary Medicine, for statistical help and Professor Aykut ÖZKUL from Ankara University, Faculty of Veterinary Medicine, for supplying Tu00 PPRV isolate for polyclonal antibody production. This manuscript is summarized from Kirikkale University, Institute of Health Science PhD thesis.

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Accepted July 8, 2011