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ARTICULOS ORIGINALES

Bovine respiratory syncytial virus in-situ hybridization from sheep lungs at different times postinfection

Hibridación in situ del virus respiratorio syncytial bovino en pulmón de cordero a diferentes tiempos postinfección

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Resumen

Se estudió, mediante la técnica de hibridación in situ, la distribución del ARN viral del virus respiratorio Sincicial Bovino (VRSB) en pulmón de corderos infectados en forma experimental, a

corderos de raza Merino de ambos sexos y de un peso vivo de 55 (+/- 10) Kg, fueron inoculados por vía intratraqueal con 40 ml de solución salina que contenía 1.26 x 10^6 DIM_{50} por ml (cepa viral NMK7). Los corderos se sacrificaron los días 1, 3, 7, 11 y 15 postinoculación. Las células epiteliales bronquiales y bronquiolares resultaron positivas a los ácidos nucleicos de VRSB los días 1, 3, 7 y 11 postinoculación. A su vez, el epitelio alveolar contenía células positivas los días 1, 3, y 7 postinoculación. Se detectaron células que contenían el ARN viral en las luces bronquiales y bronquilares del día 1 al 11 postinoculación, y en el exudadado alveolar en los días 3 y 7 postinoculación. Se identificaron señales positivas de hibridación desde el día 3 al 11 postinoculación, tanto en las células intersticiales mononucleares como en el tejido linfoide asociado a los bronquios. Las señales de hibridación más intensas se detectaron a los días 3 y 7 postinoculación, lo que coincidió con las lesiones histopatológicas de mayor consideración.

Palabras claves: Virus respiratorio sincicial bovino, hibridación, oveja, pulmones.

Summary

We studied the distribution of bovine respiratory syncytial virus (BRSV) RNA in lungs of experimentally infected sheep by in situhybridization at different times postinfection. The probe used for in-situ hybridization was prepared by reverse transcription of BRSV RNA, followed by PCR amplification of the cDNA. Twenty five Merino sheeps of both sexes with a live weight of 55± 10 Kg, received a intratracheal inoculation of 40 ml saline solution containing 1.26 x 10^6 $TCID_{50}$ BRSV (strain NMK7) per ml. Sheep were slaughtered 1,3,7,11 and 15 postinoculation days (PID). Bronchial and bronchiolar epithelial cells were positive for BRSV nucleic acid by ISH at 1, 3, 7 and 11 PID. However, alveolar epithelial cells contained positive hybridization signals cells at 1,3 and 7 PID. Cells containing viral RNA were detected from 1 to 11 PID, in exudate within bronchial and bronchiolar lumina; and from 3 to 7 PID in alveolar exudates. Positive hybridization signals were identified in interstitial mononuclear cells and in bronchi associated lymphoid tissue from 3 to 11 PID. The highest signal intensity of positive cells were observed at 3 and 7 PID, coinciding with high virus antibodies titres and with the most important histopathological findings.

Key words: bovine respiratory syncytial virus, in situ hybridization, sheep, lungs.

INTRODUCTION

Respiratory syncytial virus (RSV) is a respiratory tract pathogen of both humans and cattle. Human RSV is the most important cause of viral lower respiratory tract disease in infants and young children (Bruhn and Yeager, 1977). Bovine and human RSV strains (enveloped RNA virus) are pneumoviruses of the Paramyxoviridae family and are currently considered to be antigenically related (Thomas *et al*., 1984). Although greater suceptibility has been reported in cattle (Castleman *et al*., 1985; Bryson *et al*.,1988; 1991a,b; Kliman et al., 1989; Oberst *et al*., 1993), BRSV may also affect other domestic species, particularly small ruminants (Al-Darraji *et al*., 1982; Bryson *et al*., 1988; Masot *et al*., 1993 a,b; 1995; 1996; Redondo *et al*., 1994; Belknap *et al*., 1995). The RSV fusion protein causes fusion of viral and host cellular membranes and cell to cell fusion (Lerch *et al*., 1991).This protein is synthetisized as F_0 precursor, which is proteolitycally cleaved into two disulfide-linked polypeptide subunits, F_1 (48KDa) and F_2 (20KDa), that form the active fusion protein (Walsh *et al*., 1985; Walravens *et al*., 1990; Lerch *et al*., 1991; Paton *et al*., 1992; Pastey and Samal, 1993; Matheise *et al*., 1995). The BRSV F mRNA is 1899 nucleotides in length (Lerch *et al*., 1991). The nucleotide sequence encoding the F protein is highly conserved (>95%) among BRSV strains (Pastey and Samal, 1993). This identity is lower between F mRNAs of RSV of bovine and human origin (71.5%) (Lerch *et al*., 1991).

A digoxigenin-labeled DNA probe corresponding to the part of the genome that codes for bp 736-1522 of the BRSV protein gene was developed for localization of virus in lung sections. The aim of the present study is to analyze the pathology of sheep experimentally infected with BRSV, and to visualize specific hybridization signals for this virus in lung samples.

Animals and BRSV inoculum. Twenty-five gnotobiotic merino sheep of both sexes with a live weight of 55 ± 5 Kg, each received a intratracheal inoculation of 40 ml of the NMK7 strain of BRSV (supplied by Dr. Gómez-Tejedor, Instituto Nacional de Investigaciones Agrarias, Madrid, Spain) diluted to a concentration of 1.26 x 10^6 $TCID_{50}$/ml. The origin and characterization of the NMK7 isolate of BRSV has been previously reported by Belknap *et al*., (1995). The sheep were housed in an isolation barn, and were euthanized 1,3,7,11 and 15 postinoculation days (PID).

Virus inoculum was prepared using primary bovine fetal kidney (BFK) cell cultures infected with a BRSV stock strain NMK7, to which minimun Essential medium (Sigma, Madrid, Spain) with 15% bovine fetal serum (Sigma, Madrid, Spain) was added; the final cultures were incubated at 37ºC in a CO_2 atmosphere. Ten animals used as control were inoculated with an identical volume of un-infected BFK cell culture. They were kept in another isolation box and were slaughtered 1,3,7,11 and 15 PID.

Aliquots of virus were cultured and were determined to be free of aerobic bacteria and mycoplasma by standard techniques.

Samples of lung were collected for microbiological studies and virus isolation. Formalin fixed section were used for histopathological techniques and in-situ hybrydization assay.

Histopathology: Sections, 3 µm thick, were cut and stained with haematoxylin and eosin for routine morphological studies.

Immunoperoxidase staining procedure for detection of BRSV antigens. An Avidin-Biotin- Peroxidase complex was carried out on deparaffined and trypsinized lung samples. Sections were blocked in diluted (1:50) normal swine serum (Dakopatt, Spain) for 15 minutes to reduce background and were incubated in diluted (1:1000) rabbit anti-RSV (Dakopatt, Spain) for 3 hours at 20ºC. Diluted (1:500) biotinylated swine anti-rabbit IgG (Dakopatt, Spain) was placed on the sections for 30 minutes, followed by 1 hour incubation in diluted (1:50) ABPC reagent (Dakopatt, Spain). Sections were incubated in diaminobenzidine solution for 5 minutes and were counterstained lightly with Mayer´s haematoxylin. The positive controls included BRSV-infected BFK cell cultures (the cytopathic effect was evident in the formation of numerous syncytia) and noninfected BFK cell cultures. Test control sections were also stained using nonimmune rabbit serum as first layer.

Microbiological investigations. Lung samples tissue were homogenized in phosphatebuffered saline and a serial 10-fold dilutions were made up to 10^4 and 0.1 ml of each dilution spread on to half a sheep´s blood agar plate. Duplicate plates were used, one incubated aerobically and the other anaerobically under H2 containing 10% CO_2. Colonies were identified using the methods described by Cowan (1974). Bronchial swabs and lung samples were also cultured for mycoplasma in modified Hayflick´s broth, and colonies were identified on agar plates. Nasal swab specimens and lung samples were used to detect BRSV by virus isolation in BFK cells, following the technique described previously by Castleman *et al*., 1985. Isolates were confirmed as BRSV by indirect immunofluorescence (Masot *et al*., 1993a).

RNA extraction. BT cells (75 cm^2 flasks) were infected with BRSV strain NMK7 (Lerch *et al*., 1991; Oberst *et al*., 1993); provided by Dr. Kelling, Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, USA. This is a BRSV strain plaque purified more than three times that was originally obtained from cattle and is bovine viral diarrhea (BVD) virus free (Lehmkuhl *et al*., 1979). Cell cultures were incubated at 33 ºC in high glucose Dulbecco modified Eagle medium (DMEM) supplemented with 2% horse serum. Viruses were allowed to replicate and form syncytia (cytopathic effect), at which point the RNA was extracted from infected cells. RNA was isolated with the Trizol LS Reagent (Gibco, NY, USA) following the manufacturer's instructions. RNA concentration of the extracts was estimated by measuring the A_{260} with a Beckman DU-64 Spectrophotometer.

RT-PCR. An amplified product of 786 bp lenght encoding for bp 736-1522 of the BRSV protein F gene, was obtained by nested RT-PCR. Primer 1555 (5' TTTCCTACATCTACACTG 3') was used to obtained cDNA and primers 736 (5' TGGTATTACCACACCCCTCAG 3') and 1522 (5' TCAGATCGACGTATGAAAGCC 3') were used for PCR. The PCR reaction was performed in 35 cycles of 1 min at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC.

(Invitrogen, CA, USA). The correct sequence was confirmed by sequencing. The probe was prepared by removing the DNA insert by digestion with restriction enzyme *Eco*RI. The DNA was submited to gel electrophoresis and the fragment extracted using Quiaex II (Quiagen, Hilden, Germany). DNA fragment was digoxigenin-labeled by random priming (Boehringer Mannheim Biochemicals, Mannheim, Germany). The specifity of the probe was checked by dot blot and southern blot onto nylon membranes.

Preparation of samples for ISH. BRSVinfected and uninfected cell cultures were used for optimization of ISH. BT cells were infected and grown as has been described before . The cultures that showed cytopathic effect were trypsinized and washed twice with DMEM. The first time with the DMEM containing 5% equine serum and the second time containing as serum. After counting, the harvested cells were applied to ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA) by centrifugation at 1500 rpm for 3 min (cytocentrifuge Shandon Cytospin 2) at a density of $2x10^5$ cells per spot. Slides were fixed in 4% paraformaldehyde and dehydrated through an ethanol series.

Lung sections, 3 µm thich, were deparaffinized with xylene and rehydrated through decreasing concentrations of ethanol. All the samples (slides containing cells or sections) were equilibrate in phosphate-buffered saline (PBS). Permeabilization to allow the penetration of the probe was carried out by treatment with 0.2N HCl for 20 min at room temperature and digestion with 5 µg/ml of proteinase K (Promega, Madison, WI, USA) for cells and 30 µg/ml for tissues in PBS for 20 min at 37ºC. Some slides were treated with 50 µg/ml of RNase (Boehringer Mannheim Biochemicals) in PBS for 60 min at 37ºC in wet chamber, to be used as specificity control. Next, slides were refixed in 5% paraformaldehyde and followed by two rinses in PBS. Slides were acetylated in 100 mM triethanolamine with 0.25% of acetic anhydre (to neutralize positive charges and thus reduce nonspecific electrostatic binding of the probe) for 10 min. Slides were then washed twice in 2x SSC (1x SSC is 15 mM of sodium citrate plus 0.15 M NaCl, pH 7.0).

Prehybridization. Slides were prehybridized at 70ºC for 60 min in a humidified chamber by adding 200 µl of hybridization solution containing 4x SSC, 2mM EDTA, 10% dextran sulfate, 50% deionized formamide, 500 µg of salmon sperm DNA and 1x Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin and 0.02% Ficoll 400) per ml, without cDNA probe.

Hybridization. The ISH conditions were as described elsewhere (Sur *et al*., 1996).

Enzyme-Linked Immunoabsorbent Assay (ELISA). Positive control antigen was prepared from bovine foetal kidney cell cultures infected with BRSV strain NMK7. Negative control antigen was determined by block titration; those which yielded maximum optical density (OD) values for positive sera without causing an increased OD in negative sera were considered satisfactory.

Polystirene plates were coated with 100 µl of the antigen diluted to a concentration of 5µg/ ml in sodium carbonate buffer (0.1 M, pH 9.6) with 0.02% NaN3 for 3 h at 37 ºC. After coating and between all subsequent steps, the plates were washed 4 times in phosphate-buffered saline containing 0.05% Tween 20 (PBSTw). As a blocking agent, 5% bovine serum albumin (BSA) in washing buffer was added (200ml) and incubated for 30 min at 37 ºC. After washing, serum samples diluted to 1/1000 in PBSTw (100 µl) were added (200ml) and incubated for 30 min at 37 ºC. After subsequent washing, 100 µl of horseradish peroxidase conjugate mouse antisheep IgG (diluted 1/2000 in washing buffer) were added. The reaction was developed at room temperature by the addition of 100 µl of 0.04% ophenylenediamine and 0.004% H_20_2 in citratephosphate buffer (pH 5), stopped after 1h by the addition of 50 µl of 3N H2 SO4 and read on a spectrophotometer at 490 nm.

The O.D. of BRSV = O.D of BRSV Ag – O.D. Control Ag.

Light Nephelometry. A biological fluid protein counter was used to determine immunoglobulin levels. This system measures the intensity of light and the dispersion of the light beam as it passes through particles in cell fluid suspension. Particles were formed by immunoprecipitation of diluted (1:100) experiment sera with diluted (1:50) monoclonal sheep anti-IgA, anti-IgG and anti-IgM antibodies.

RESULTS

BRSV inoculum. Viral inoculum was free of aerobic bacteria, mycoplasma and BVD virus.

Histopathology. The histopathological study has been previously reported by us on studies reported here (Masot *et al*., 1995, 1996). Catarrhal bronchiolitis observed on 1 PID was associated with granulocyte infiltration of the bronchiolar lumen. The interalveolar septa were thickened, with pronounced interstitial edema and moderate cell reaction.

Alveolar exudate in animals slaughtered 3 and 7 PID consisted of neutrophils, lymphocytes and multinucleate giant cells which formed the syncytia. There was considerable thickening of the interalveolar septa, due to the presence of edema and granulocyte and monocyte infiltration. Bronchiolitis was accompained by epithelial cell necrosis. Hyperplasia of bronchiolar epithelial cells was conspicuous and early stages of reepithelization were apparent. Exudate in the bronchiolar lumina consisted of desquamated cells, necrotic debris and syncytial cells.

Severe bronchial, bronchiolar and alveolar damage was visible by 11 PID. Lung parenchyma had clear focal areas of consolidation due to bronchiolitis obliterans and the alveolar collapse caused by infiltration of macrophages, lymphocytes and syncytial cells into lumina. Animals slaughtered at 15 PID presented a marked interstitial inflammatory reaction, with considerable septal thickening. Intense bronchiolar and/or alveolar hyperplasia was also observed. Lung consolidation was less marked than in animals slaughtered earlier.

IHC signals. The IHC study has been previously published by us on studies reported here (Masot *et al*., 1993b, 1996). BRSV antigen was detected in bronchial and bronchiolar epithelial cells, in bronchial mucous cells and in alveolar epithelial cells at 3 and 7 PID. Intense staining was also observed in alveolar macrophages, interstitial mononuclear cells and syncytia from 3 to 11 PID. Antigen was commonly detected in exudate within bronchiolar, bronchial and alveolar lumina. Specific staining was absent in the negative control (table 1).

Microbiological findings. Twenty three of the sheep inoculated with BRSV and nine of the control animals, were bacteriologically negative at the conclusion of the experiment. Contaminative species of bacteria were E. coli and Bacillus spp. Mycoplasma was not isolated from the upper or lower respiratory tract of any sheep.

ISH signals. In all PID the specificity of the signal reaction was confirmed by the rigorous observance of specific controls (figure 1, 2). The BRSV nucleic acid signal was specific because this was completely absent from non infected cells (figures 1, 2). There was no detectable ISH signal of BRSV RNA in the lungs of the control sheep (table 1).

Hybridization signals specific for BRSV RNA were detected in bronchial and bronchiolar epithelial cells at 1,3,7 and 11 PID (figure 3 and 4) but they were not observed at 15 PID.

BRSV-positive cells was commonly detected in exudate within bronchial and bronchiolar lumina from 1 to 11 PID (figure 5).

The positive IHS signal was observed in alveolar epithelial cells at 1, 3 and 7 PID (figures 6, 7, 8). In several cases the signal of RNA-virus also coincided with a cell morphology and location that would suggest that the cell could be classified as a type II pneumocyte. The RNA genome was visible in: free cells in alveolar space (figure 9) and in syncitial alveolar cells (figure 10) at 3 and 7 PID.

Hybridization signal specific for BRSV RNA was observed in the peribronchiolar area (figures 11,12), in bronchus associated lymphoid tissue (figure 12) and in mononuclear cells of interalveolar septum from 3 to 11 PID.

ELISA. Serum IgG BRSV antibody titres are summarised in figure 13. A progressive increase in titres was recorded until 7 PID; giving way, to an decrease from 11 PID onwards. The highest antibody response was observed in PID 3 and 7.

Light Nephelometry. Serum IgA, IgM and IgG levels are shown in table 2. The maximun

those recorded for preinfected animals throught the experiment.

Viral isolation
Results of BRSV isolation attemps from lung homogenates or nasal secretions are presented in table 2.

DISCUSSION

The positive IHS signal in bronchi and bronchioles is indicative of the direct pathogenic action of BRSV at these levels (Viuff *et al*., 1996) inducing necrosis and desquamation of bronchial and bronchiolar epithelia. Necrosis of bronchial and/or bronchiolar epithelia has been described elsewhere as a limitation of pulmonary antibacterial defenses with impaired mucociliary clearance, and as a predisposition to secondary bacterial infection (Pirie *et al*., 1981; Al-Darraji *et al*., 1982; Castleman *et al*., 1985; Bryson *et al*., 1988; Redondo *et al*., 1994; Masot *et al*.1993 a,b; 1995; 1996; Viuff *et al*., 1996).

Cells obstructing bronchial and bronchiolar lumina tend to form syncytia (Bryson *et al*.,1988; 1991a,b; Ciszewski *et al*., 1991; Masot *et al*., 1993a,b; 1995; 1996; Belknap et al., 1995) which are usually located in bronchioles and rarely observed in bronchi. This finding has previously been reported in studies of immunohistochemistry (Masot *et al*., 1993a,b) or in situ hybridization (Viuff *et al*., 1996). The possible involvement of type I and II pneumocytes in BRSV replication in sheep lung has been detected by IHC (Masot *et al*., 1993a,b, 1995, 1996). Extensive hypertrophy and hyperplasia of type II pneumocytes are also known to occur during acute BRSV infection in the lung (Masot *et al*., 1993a, 1995, 1996). In the present study, results with IHS bears out the role of type II pneumocytes in BRSV replication, since the lung section showed cells exhibiting positive IHS signals and which were morphologically and topographically consistent with type II pneumocytes. In this connection,Viuff *et al*., (1996), in natural infected calves, suggest that the replication of BRSV in the alveolar type II cells, may lead to a change in the amount and quality of surfactant. The deficiency of surfactant caused by BRSV replication in the type II pneumocytes, may be a determining factor in collapse of alveoli. The alveolar collapse was visible in lungs of experimentally infected sheep described in the present study. In order to confirm that type II pneumocytes represent a specific BSRV replication site, TEM should be performed on the same sections as those used for IHS.

TABLE 1. IHC and ISH signal in the lung of experimentally infected sheep by BRSV.
IHC and ISH signal in the lung of experimentally infected sheep by BRSV.

		ISH								IHC							
		BEC	BE	BrEC	BrE	AEC	SYN	BALT	MC	BEC	BE	BrEC	BrE	AEC	SYN	BALT	MC
PID-10	Nº 26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PID-0	Nº 31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PID-1	Nº 1	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
	Nº 2	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
	Nº 3	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
	Nº 4	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
	Nº 5	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
PID-3	Nº 6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PID-7	Nº 11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Nº 15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PID-11	Nº 16	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
	Nº 17	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
	Nº 18	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
	Nº 19	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
	Nº 20	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+
PID-15	Nº 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nº 25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, Lamb positive by IHC or ISH signal. - Lamb negative by IHC or ISH signal. BEC = bronchial epithelial cells; BE = bronchial exudation; BrEC = bronchiolar epithelial cells; BrE = bronchiolar exudation. AEC = Alveolar epithelial cells; SYNC = syncitial alveolar cells; BALT = bronchus associated lymphoid tissue; MO = Mononuclear cells.

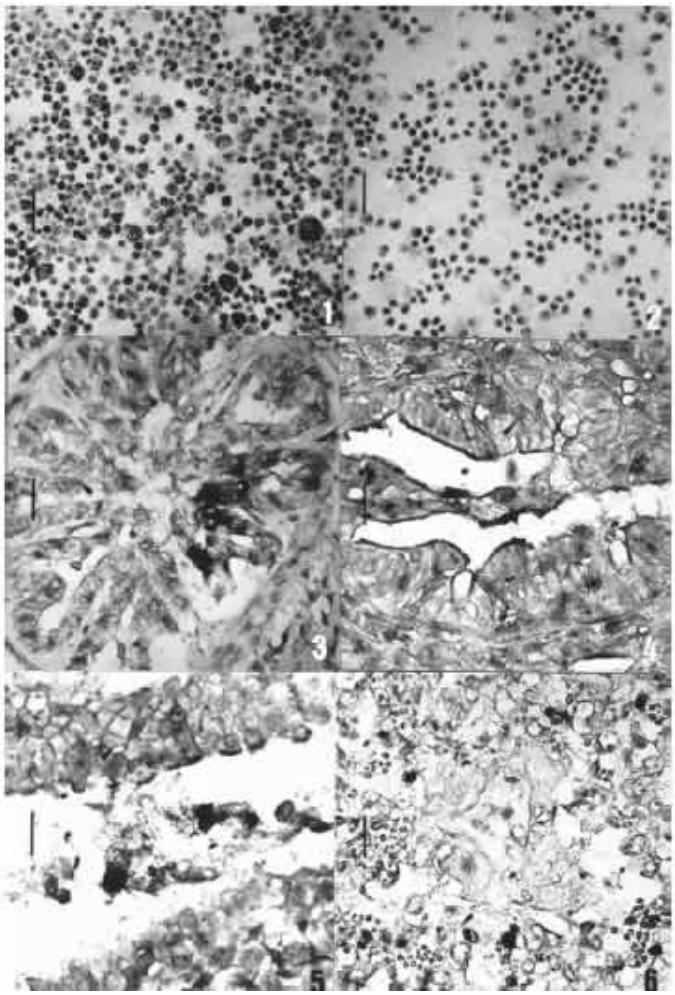

FIGURE 1. Bovine Respiratory Syncytial Virus (BRSV) infected BT cells hybridized with a DIG-labelled cDNA probe. A positive ISH signal in the cytoplasm of cells is shown. Methyl green counterstain. Bar = 160 µm.

FIGURE 2. In situ hybridization of mock-infected BT cells with the same probe. Methyl green counterstain. Bar = 160 µm.

FIGURE 3. Lung of sheep BRSV-infected at 7 days p.i. ISH positive signal in bronchiolar epithelial cells. ISH counterstained with methyl green. Bar = 80 µm.

FIGURE 4. Lung; sheep inoculated with BRSV and slaughtered at 11 PID. ISH positive signal in bronchiolar epithelial cells. ISH countersstained with methyl green. Bar = 80 µm.

FIGURE 5. Lung; sheep inoculated intratracheally 7 days previously with BRSV. BRSV RNA infected cells located in bronchiolar exudations. ISH counterstained with methyl green. Bar = 40 µm.

FIGURE 6. Lung; sheep inoculated intratracheally 1 days previously with BRSV. BRSV nucleic acid detected in alveolar epithelial cells. ISH counterstained with methyl green. Bar = 160 µm.

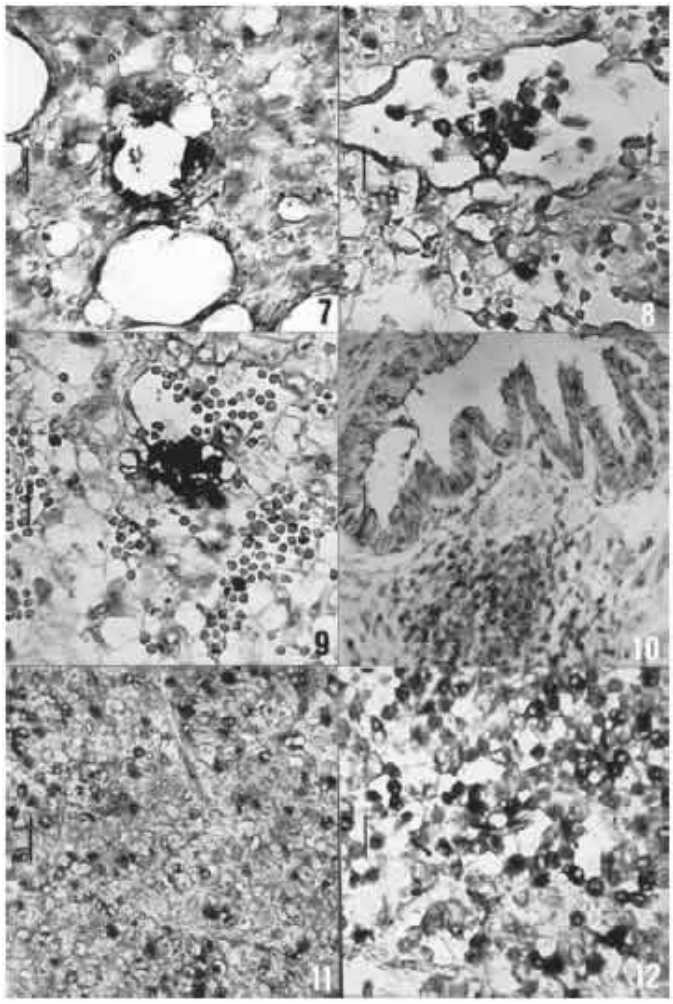

FIGURE 7. Lung; sheep inoculated intratracheally 3 days previously with BRSV.ISH positive signal observed in alveolar epithelial cells. These cells that by morphology and localization could be classified as type II pneumocytes. ISH counterstained with methyl green. Bar = 80 µm.

FIGURE 8. Lung; sheep inoculated intratracheally 7 days previously with BRSV. BRSV infected cells located in alveolar epithelium. ISH counterstained with methyl green. Bar = 80 µm.

FIGURE 9. BRSV infected lung tissue taken from an infected sheep at 3 PID. Large numbers of positive cells free in alveolar space. ISH counterstained with methyl green. Bar = 80 µm.

FIGURE 10. BRSV infected lung tissue taken from an infected sheep at 7 PID.Syncytia containing BRSV RNA positive cells. ISH counterstained with methyl green. Bar = 80 µm.

FIGURE 11. Lung; sheep inoculated with BRSV and slaughtered at 3 PID. Positive signal in the peribronchiolar area. ISH counterstained with methyl green. Bar = 80 µm.

FIGURE 12. Positive signal in the peribronchiolar area of the lung of sheep at 3 days postinoculation with BRSV. ISH counterstained with methyl green. Bar = 80 µm.

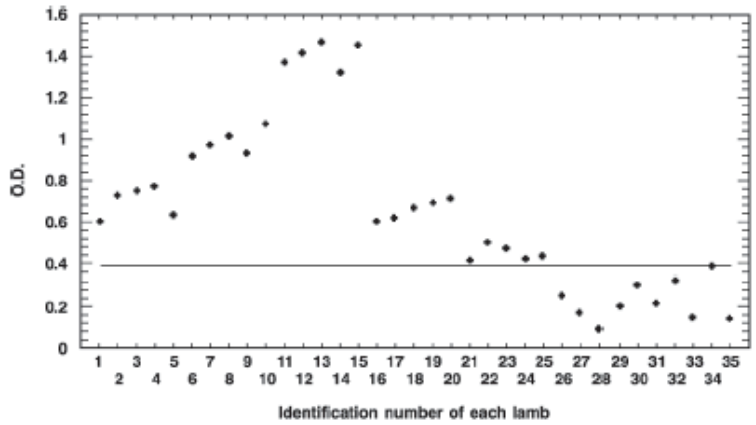

FIGURE 13. Sheep experimentally infected with BRSV. Serum IgG BRSV antibody titres detected by ELISA. PID 1 (sheep number 1-5); PID 3 (sheep number 6-10); PID 7 (sheep number 11-15); PID 11 (sheep number 16-20); PID 15 (sheep number 21-25); Control: PID -10 (sheep number 26-30); PID -0 (sheep number 31-35).

In this experiment, a positive IHS signal for BRSV in alveolar macrophages could not be established (Viuff *et al*., 1996). These results agree with previous reports (Schrijver *et al*., 1994) suggesting that in vitro bovine alveolar macrophages (BAMs) exhibit a high intrinsic resistance for infection with BRSV and that bovine alveolar macrophages do not appear to be important for replication of BRSV. However, large numbers of BAMs may harbour virus antigen, even for several days, that may influence the function of these cells (Schrijver *et al*., 1994). However, replication of human respiratory syncytial virus in alveolar macrophages has been shown (Cirino *et al*., 1993).

A large number of alveolar syncytia containing BRSV-RNA were detected. The location of these syncytia coincided with the positive hybridization signals reported by Viuff *et al*., (1996), and with the site of BRSV antigen detected elsewhere by IHC (Bryson *et al*., 1988a; Masot *et al*., 1993a,b, 1995, 1996). However, the reason for syncytia formation remains an enigma. Syncytia are probably a result of fusion of cells and a direct spread of virus from cell to cell (Viuff *et al*., 1996).

TABLE 2. BRSV isolation fron nasal secretions and lung homogenates. Means of serum immunoglobulling levels (g/dl).
BRSV isolation fron nasal secretions and lung homogenates. Means of serum immunoglobulling levels (g/dl).

BRSV ISOLATION		IgG (g/dl)	IgA (g/dl)	IgM (g/dl)
Lung	Nasal secretions			
-	-	2180	33	214
-	-	2183	35	216
-	-	2186	36	217
-	-	2190	32	220
-	-	2193	34	218
-	-	2188	35	221
-	-	2187	33	222
-	-	2183	34	221
-	-	2189	33	218

+	-	2220	33	222
+	+	2218	33	218
+	+	2216	34	221
+	+	2221	32	219
+	-	1967	36	228
+	+	1951	33	208
+	+	1957	37	211
+	+	1964	34	214
+	+	1967	32	217
+	+	1975	30	219
+	-	1980	33	224
+	+	1983	35	222
+	+	1986	36	226
+	+	1978	31	221
-	-	3245	46	212
-	-	3280	49	209
+	+	3275	51	211
-	+	3282	52	214
-	-	3269	50	204
-	-	3345	54	218
-	-	3359	51	222
+	+	3367	56	209
-	-	3298	59	216
-	-	3396	61	221

+, Lamb positive by viral isolation. -, Lamb negative by viral isolation.

In the present study, positive ISH signals were observed in mononuclear cells in the interstitial (interalveolar and peribronchial) space and in BALT of damaged areas from 3 to 11 dpi. The distribution and topography of specific hybridization signals in the interstitium was very similar to the location of BRSV antigen detected by IHC (Bryson *et al*., 1988a; Masot *et al*., 1993c, 1995, 1996). However, the site of BRSVRNA positive cells in BALT was a differentiating feature, since similar findings have not been reported elsewhere (Viuff *et al*., 1996).

Interstitial cells expressing BRSV-RNA may carry virus particles to other organs, as described elsewhere (Lerch *et al*., 1991).

The results obtained showed that more intense positive hybridization signals were detected at 3 and 7 dpi. It was precisely on these days that lesions were most intense (Masot *et al*., 1993a,b, 1995, 1996),coinciding with maximum levels of serum IgG anti-BSRV. (Masot *et al*., 1993a). However, this relationship is not clearly defined, since previous reports (Ciszewski *et al*., 1991), state that levels of anti-BSRV serum antibodies remained relatively constant from 4 to 21 dpi with BRSV.

High values for IgG (PID 11 and 15) were associated with a marked decrease of BRSV antibody titres on days 11 and 15 PI, (Korbecki and Maksymowicz, 1977). Other authors report high neutralising antibody levels in calves from 5 PID onward, although maximal levels were detected at 5 weeks PI (Elazhary *et al*., 1981; Castleman *et al*., 1985). However, other studies have found low serum IgG values in experimentally-infected calves, although this response was recorded only when the animals were exposed to the virus for second time Mohanty *et al*., 1975).A marked increse in serum IgA levels were coincident with the decrease of virus antibodies titres over the same period (Korbecki and Maksymowicz). Other studies, however, report an absence of IgA in the serum of calves experimentally infected with human and bovine RSV (Thomas *et al*., 1984).

In our experience, serum IgM levels did not increase. This finding conflicts with the increase in serum IgM levels described in children with RSV infection (Bruhn and Yeager, 1977; Korbecki and Maksymowicz, 1977).

authors were able to show, through IHC, that sites containing viral antigen corresponded to virus replication sites.

It is a well-known fact that immunodetection requires a large number of molecules in order to obtain positive reactions. In prolonged postinfection periods, the number of positive cells detected by immunohistochemistry (IHC) decreased; it was also sometimes difficult to differentiate between positive and negative signals, especially with high levels of background noise. IHC therefore has more limitations than IHS; with impaired antigen production in infected cells, viral RNA in cells persists (Schrijver *et al*., 1994).

Further studies of comparison of consecutive serial sections are necesary to stablish cell to cell correlation between the signal of both techniques.

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