Pergamon

A MODEL FOR RESPIRATORY SYNCYTIAL VIRUS (RSV) INFECTION BASED ON EXPERIMENTAL AEROSOL EXPOSURE WITH BOVINE RSV IN CALVES

P. OTTO,[1]* MANDY ELSCHNER,[1] PETRA REINHOLD,[1] HEIKE KÖHLER,[1] H.-J. STRECKERT,[2] S. PHILIPPOU,[3] H. WERCHAU[2] and K. MORGENROTH[3]

[1] Federal Institute for Health Protection of Consumers and Veterinary Medicine, Jena Branch, Box, 07722 Jena, Germany, [2] Department of Virology, Ruhr-University Bochum, 44780 Bochum, Germany and [3] Department of Pathology, Ruhr-University Bochum, 44780 Bochum, Germany

Abstract—Five conventionally kept calves aged between 17 and 24 days were experimentally infected with bovine respiratory syncytial virus (BRSV) by aerosol in order to mimic the natural infection route. The calves were killed and autopsies performed 7 days after the first virus challenge. The BRSV isolate used induced tracheitis, bronchitis and atelectasis in infected calves. The only virus which could be isolated from the lungs of the calves was BRSV. In addition, *Mycoplasma bovirhinis* was isolated from the lungs or/and trachea of two calves. The clinical and histopathological findings, as well as the detection of BRSV antigens by immunofluorescence in the epithelial cells of lung and trachea, and the reisolation of the virus from bronchoalveolar lavage fluids of all inoculated calves, provided confirmation of successful infection with BRSV.

Key words: Respiratory syncytial virus, calf, aerosol exposure.

Résumé—Afin de stimuler une infection naturelle, 5 veaux conventionnels, âgés de 17 à 24 jours, furent l'objet d'une infection expérimentale réalisée à l'aide d'aérosols contenant le virus respiratoire syncytial bovin (BRSV). Les veaux furent euthanasiés et autopsiés 7 jours après la première exposition au virus. Les isolats de BRSV utilisés provoquèrent, chez les veaux infectés, une trachéite, une bronchite et de l'atelectasie. Le seul virus pouvant être isolé des poumons fut le BRSV. *Mycoplasma bovirhinis* fut également isolé des poumons et/ou de la trachée de deux veaux. Les constatations cliniques et histopathologiques, la détection (par immunofluorescence) des antigènes BRSV dans les cellules épithéliales du poumon et de la trachée, et l'isolement du virus dans le liquide issu des lavages broncho-alvéolaires confirmèrent le succès de cette infection par BRSV.

Mots-clefs: Virus respiratoire syncytial, veau, aérosol exposition.

INTRODUCTION

Human respiratory syncytial virus (HRSV) has been accepted as a major cause of severe lower respiratory tract disease in infants and in young children, particularly in atopic patients [1–3]. Bovine respiratory syncytial virus (BRSV) is capable of producing

*Author for correspondence.

respiratory infections in cattle [4–9]. The disease is most severe in children and calves between 1 and 3 months of age [10]. Both viruses are antigenically and biochemically closely related [11]. Furthermore, infections by these viruses show many common features regarding clinical disease and pathology [12].

Laboratory animals, which have been used extensively to study the pathogenesis and immunopathogenesis of RSV infections, have certain disadvantages. Firstly, they are not naturally infected by RSV, therefore the cotton rat model of HRSV infection does not produce clinical signs [13]. Secondly, there is a large variation in the results, i.e. between ferrets and cotton rats [14, 15]. Furthermore, the laboratory animals do not allow the introduction of methods of lung function testing *in vivo*.

Because of the similarities between HRSV and BRSV and their respective diseases, cattle are the best natural host model available for studying the pathogenic mechanisms of RSV infections. Also, the BRSV infection in calves is thought to be a more appropriate model for human RSV infection of children than human RSV infection of laboratory animals because calves, as well as human infants, may become infected by RSV even in the presence of passively derived antibodies. Moreover, the calf model allows extensive lung function studies *in vivo*, which are currently under way. In most cases a combined intranasal/intratracheal infection route was successful in producing respiratory tract disease and lesions [16, 17–21]. However, an appropriate validation of the effects of such severe artificial infections is difficult, because it may reflect little of what occurs under natural conditions. The spread of HRSV may occur by close contact with direct inoculation of large droplets or by self-inoculation after touching contaminated surfaces, but the infection did not occur if the particles had to travel distances of 2 or more metres [22].

The aim of the present study was to establish a BRSV infection model in conventionally reared calves which worked with exposure doses administered by aerosol route via face mask.

MATERIALS AND METHODS

Calves

Nine clinically healthy Friesian crossbred, colostrum-fed calves (6 male, 3 female) were obtained from a local dairy herd and were conventionally reared. The animals were bedded on straw and housed in a ventilated calf house. They were fed twice daily with a commercial milk replacer and received hay. Each calf was allowed to adjust to the new environment for 1 week. During this time, daily clinical investigations were done. Five animals (Nos. 1–5) were exposed to the virus at the age of 17–24 days. Four calves (Nos. 6–9) were similarly inoculated with noninfected tissue culture cells at the age of 21–25 days.

Virus and cell culture

BRSV 375 isolate (ATCC No.: VR-1339) was propagated in bovine turbinate (BT) cells maintained in Eagle's MEM to which 1% BMS (serum replacement: Biochrom KG, Berlin) and gentamycin (50 μg/ml) had been added. Cultures were incubated at 37°C in 5% CO_2. The medium was usually changed after 48 h. Virus was harvested by scraping the cells into the culture medium 6–8 days p.i. and stored at -80°C until infection of calf No. 1. Aliquots of the virus were cultured and were determined to be free of mycoplasma, ureaplasma and bovine viral diarrhoea virus (BVDV) by standard techniques. The BRSV titer was determined in a titration assay with immuno enzymatical detection. Briefly,

10^4 BT cells in $100 \,\mu$l of medium were seeded into the wells of a 96 well tissue culture plate. The appropriately diluted virus suspensions in medium ($100 \,\mu$l) were added. At 48 h p.i. the cells were fixed with ethanol and analysed for the presence of RSV proteins using a rabbit anti-RSV hyperimmune serum (1:3000 diluted in phosphate buffer). Enzyme-linked labelling of bound antibodies was performed by means of a biotinylated anti-rabbit Ig antibody (Dako Diagnostika, Hamburg, Germany) and subsequent incubation with extravidin peroxidase (Sigma, Deisenhofen, Germany). 3-Amino-9-ethylcarbazole (Sigma) was used for enzyme reaction.

Since the BRSV isolate could be reisolated from the bronchoalveolar lavage (BAL) fluid of the first infected calf, this reisolated virus was used further for the infection of the following four calves.

For the isolation of other viruses than BRSV after necropsy, primary or secondary bovine kidney (BK) cells were prepared in Hanks balanced salt solution to which lactalbuminhydrolysat (5.0 g/l), 10% neonatal calf serum and gentamycin ($50 \,\mu$g/ml) had been added. For reisolation of BRSV, BT cells were prepared as described above in medium containing 10% inactivated foetal calf serum. After inoculation of the mono-layers, the BK cells were maintained without serum and the BT cells with 1% BMS.

Aerosol exposure

Approximately 180 l aerosol per day per calf was produced from 4.5 ml of noninfected culture fluid or culture fluid containing 10^4–10^5 PFU/ml of virus using a jet nebulizer (Pari Provokationstest I, Medanz, Starnberg, Germany). Each calf had to inspire the virus-containing aerosol using a tightly fitting face mask (Fig. 1). A mouthpiece with respiration valves was used in order to filtrate the expiratory flow for virus retention. The aerosol, which contained more than 60% particles smaller than $3 \,\mu$m, was administered to each calf over a period of approx. 50 min per day on 4 (calves Nos. 1, 2, 7 and 8) or 2 (calves Nos. 3–6 and 9) consecutive days, respectively.

Monitoring and sample collection

Rectal temperature, respiratory rate (RR) and other clinical findings were recorded twice daily before and after the experimental infections. Nasal swab specimens were collected with small sanitary tampons from the day before initial virus inhalation up to the end of the experiment with the exception of days 4 and 5 after infection. Serum specimens were taken prior to the first infection, and at necropsy, for estimation of antibodies against BRSV, parainfluenza virus 3 (PIV3), bovine coronavirus (BCV), BVDV and bovine herpesvirus type 1 (BHV-1).

Calves were killed 7 days after the initial exposure as follows: under conditions of deep anaesthesia (thiamylal-natrium, 1 g per 50 kg body weight intravenously), the trachea was exposed by dissection. Large arterial forceps were applied to clamp the trachea and thus to prevent a contamination of the airways by aspiration of blood or gastric contents. Following this, the animal was exsanguinated and the lung removed. Immediately thereafter lung lavage fluid was obtained from consolidated and normal-appearing right lung lobes for reisolation of BRSV and cytologic examination by instillation of 100 ml sterile PBS-buffer in 20 ml aliquots and reaspiration into the syringe with light suction (five single lavages per lavaged lung segment).

Tissue samples were taken from lesions and from macroscopically unchanged areas from right lung lobes, trachea and bronchial lymph nodes for isolation of viruses and bacteria.

The complete left lung was perfused with formaldehyde–glutaraldehyde-fixation-solution (4% formaldehyde, 2.5% glutaraldehyde in phosphate buffer, pH 7.0) for histopathologic examinations.

Indirect immunofluorescence

Frozen sections of trachea, bronchial lymph nodes, and unfixed right lung lobes were processed for immunofluorescence as described previously [23]. The characterized MAb 3C4 [23] directed against the P protein of HRSV (strain Long) was tested for specific immunofluorescence in BRSV 375 infected BT cells prior to the reported experiments (data not shown).

Virus isolation

BAL fluid of necropsied calves was centrifuged at $400\,g$ for 10 min. The supernatant (0.5 ml) was inoculated on monolayers of BK cells and BT cells. The cultures were incubated at 37°C in 5% CO_2 for up to 11 days. They were examined daily for evidence of a cytopathic effect. After three passages the replication of PIV3 in BK cells was controlled by a standardized haemagglutination assay using guinea pig erythrocytes [24]. Moreover, the haemagglutinating activity due to BCV was examined in BAL fluids using a similar haemagglutination assay (HA) with mouse erythrocytes. BAL fluid was examined for BVDV infection on primary bovine testicle cells [25]. BHV-1 was detected by an antibody virus neutralization reaction [26].
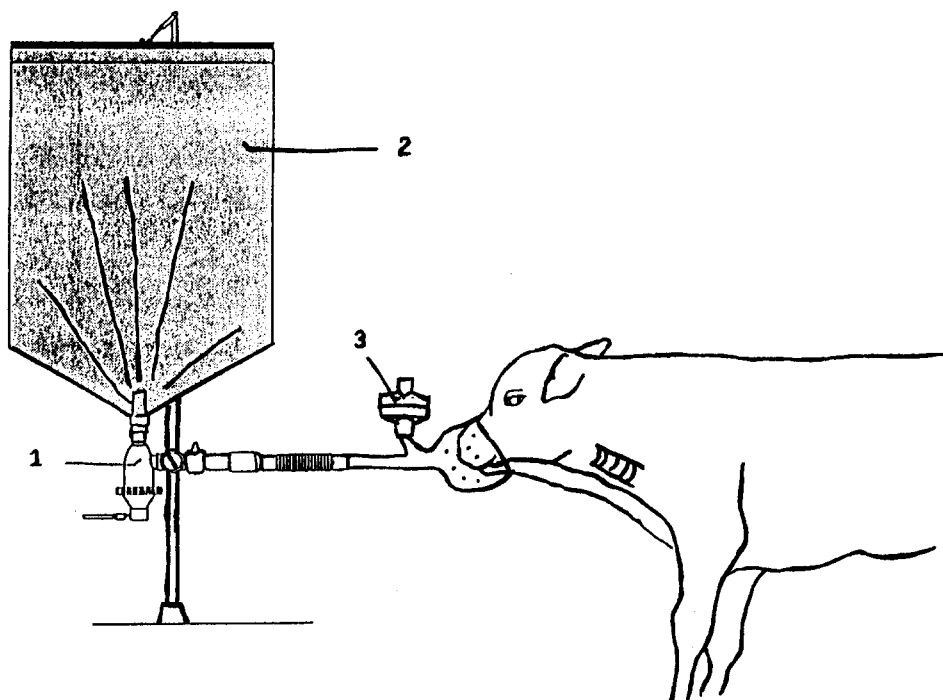

Fig. 1. Scheme for exposure to the virus-containing aerosol. 1, jet nebulizer; 2, aerosol reservoir (10 l); 3, filter for virus retention in the expiratory flow.

Bacteriological examinations

Nasal swabs, trachea and lung samples were taken and bacteriologically cultured on blood agar for aerobic bacteria. The colonies were identified by carrying out the recommendations of Iordache *et al.* [27]. Mycoplasmas were isolated and identified according to the recommendations of Freundt [28] and Gourlay and Howard [29]. Also, the lungs were examined for the presence of other mycoplasmas, such as *M. dispar* or ureaplasmas.

Serological examinations

Serum specimens of all calves were examined for antibody titers to BRSV, PIV3, BVDV, BHV-1 and BCV. The determination of BRSV antibodies was done by the above-described microneutralization assay [30]; antibodies to PIV3 and BCV were determined using a standardized haemagglutination inhibition assay (HAIA) [24]. Guinea pig erythrocytes were used for detection of PIV3 antibodies, and mouse erythrocytes for BCV antibodies. Antibodies to BVDV were determined by using a direct neutralizing peroxidase-linked antibody assay [31] and BHV-1 antibodies were determined by a serum neutralization test [32].

RESULTS

Clinical signs

A few days after the first virus exposure, a mild clinical disease could be observed in all infected calves (Nos. 1–5) without a marked increase in rectal temperature. As demonstrated in Fig. 2, mainly local appearing respiratory signs were observed characterized by serious nasal discharge, oculo-secretions, and cough. Clinical signs of respiratory disease were not observed in three control calves. On days 2, 3 and 5 after the first exposure of uninfected BT cells in calf No. 6 a mild cough could be observed. On day 2 after infection, a significant increase in the mean respiratory rate was observed in the infected calves (Fig. 3). The clinical picture did not differ between calves exposed for 2 or 4 consecutive days, respectively.

Macroscopical findings

At necropsy, atelectases were observed in four infected calves (No. 1, 2, 4 and 5). The lung lesions made up 2–8% of the pulmonary surface and were characterized by red, consolidated areas mostly located in central and subpleural parts of all lobes (Fig. 4). The visceral pleura was smooth and shiny. With the exception of calf No. 1, a copious mucoid exudate was noticed in the central airway system. The lesions were more severe in the calves No. 1 and 2 exposed for 4 consecutive days.

Control calves did not show any macroscopical or histological lesions in their lungs.

Histological lesions

In light microscopy the macroscopically visible atelectases were mainly lobularly distributed. A collapse of the bronchioli and alveoli was found. The lumen of the bronchioli was filled with desquamated epithelial cells and neutrophil granulocytes. The bronchial epithelial cells were swollen and the ciliary lining was damaged. The intercellular spaces became wide. In the bronchial epithelium and in the subepithelial zone neutrophil granulocytes were visible. In the bronchiolar wall a variably dense lymphocytic infiltrate was found (Fig. 5).
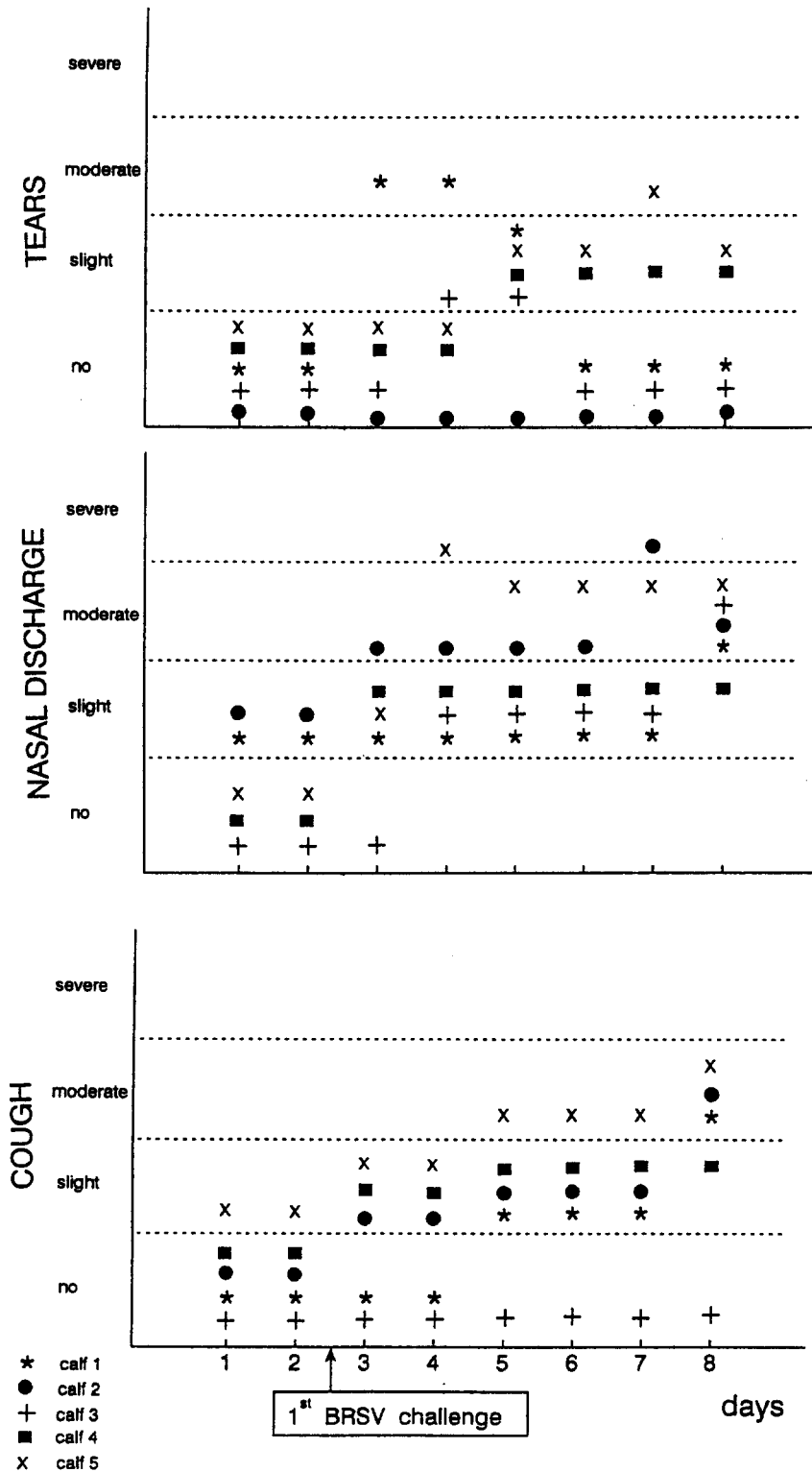
Fig. 2. Appearance of tears, nasal discharge, and cough two days before and six days after experimental infection with bovine respiratory syncytial virus (BRSV) in infected calves.

Indirect immunofluorescence

Viral antigen was present in the cytoplasm of epithelial cells of the collapsed bronchioli and in the vicinity of intraluminal cellular debris. The pattern of immunofluorescence consisted in a point-like reactivity, in part with distinct round fluorescence (Fig. 6). Bronchial epithelium with no signs of inflammation and bronchial epithelium from control calves showed a negative reaction.

Viral antigen was also detected in the tracheal epithelium; however, the reaction was much weaker than in the collapsed bronchioli. Viral antigen appeared in the form of small foci of fluorescent cells in the epithelium.

Viral and serological examinations

During the 1st passage in BT cells, the BRSV could be reisolated from BAL fluid of all infected calves. The cytopathic effect due to BRSV was obtained usually 3–4 days after inoculation (Fig. 7). The HA with erythrocytes from guinea pigs and mice was negative for BAL fluids of all calves. PIV3 and BCV were not recovered on BK cells from the BAL fluid of any calves. All BAL fluids were negative for BVDV and BHV-1. All calves had serum antibody titers to BRSV, PIV3, BCV, BVDV and BHV-1 before BRSV infection but did not develop elevations in these titers during the course of the experiment.
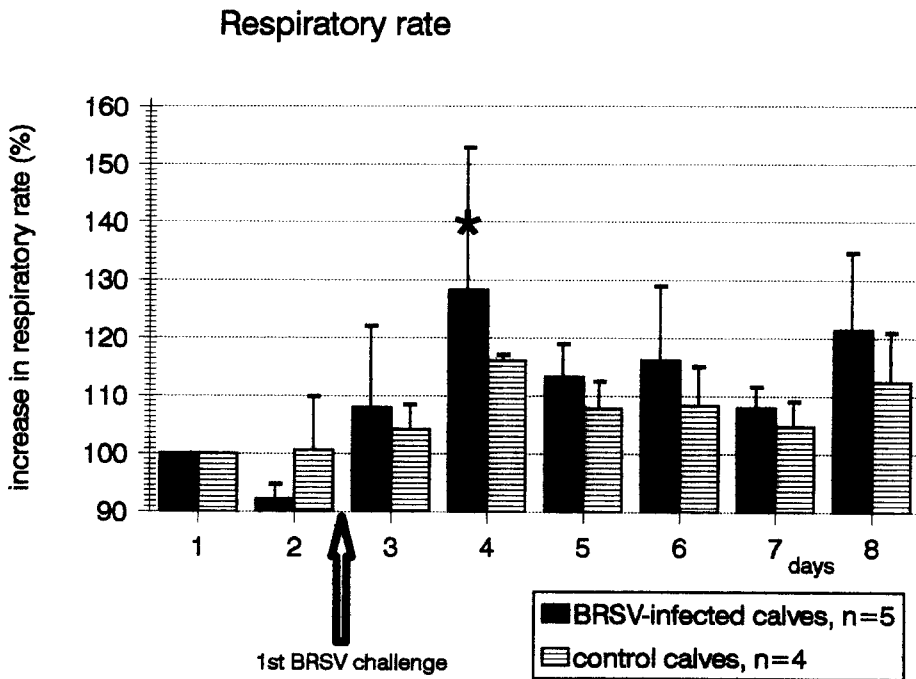

Fig. 3. Increase in respiratory rate (%) in BRSV-infected and control calves (mean, SEM). *, Significant increase in respiratory rate in BRSV-infected calves; analysis of variance test: least significant difference, $P < 0.05$.

Bacteriological findings

Before the first infection in the nasal swab of calf No. 1, *Mycoplasma* (*M.*) *bovirhinis*, and in the nasal swab of calf No. 4 *M. bovirhinis* and *Acholeplasma* (*A.*) *laidlawii*, were isolated. In the course of the experiment *M. bovirhinis* was detected in nasal swabs of six calves (Nos. 1, 3–5, 7 and 9). Moreover, *M. bovirhinis* was isolated from the lung and trachea of calf No. 1 and from the trachea of calf No. 5. *M. arginini* was isolated from nasal swabs of calf No. 6 and *A. laidlawii* was found in nasal swabs of calf No. 8. One day before the first infection in the nasal swabs of calves No. 2 and 3 *Pasteurella* (*P.*) *multocida* (capsular type A) was detected. During the infection period *P. multocida* (capsular type A) was isolated from nasal swabs of three calves (Nos. 1, 2, 5). In nasal swabs of one calf (No. 4) *P. multocida* (capsular type A or D) could be isolated. *P. haemolytica* A1 and A12 were detected in nasal swabs of calf No. 7 and *P. haemolytica* A7 was isolated from nasal swabs of calf No. 8. *Pasteurella* spp. were not recovered from lung or trachea of any calves.

DISCUSSION

Most of the studies about experimental BRSV infections in calves have involved intranasal and/or intratracheal routes of virus inoculation [16, 18–21]. In the present study we used inhalation of a virus-containing aerosol for a defined time. This kind of exposure should be closer to the natural way of infection than intratracheal virus instillation, although investigations in humans have suggested that the spread of RSV may occur by close contact direct inoculation of large droplets or by self-inoculation after touching contaminated surfaces [22]. Due to the knowledge that particles smaller than 5 μm reach the lower respiratory tract as well as alveoli in general, the main part of virus was assumed to be deposited in the peripheral parts of the respiratory system in the infected calves.

The results of the present study indicate that experimental aerosol exposure of colostrum-fed calves with BRSV resulted in the production of clinical signs and respiratory tract lesions. These findings are in agreement with previous results [16, 19]. Clinically, the BRSV strain 375 produces mild cough, serious nasal discharge, and oculo-secretions. In infected calves a significant increase in mean respiratory rate was already observed on day 2 after inoculation. Others have reported a significant increase in mean respiratory rate in colostrum-deprived, but not in colostrum-fed calves [16].

The gross and histopathological changes of calf No. 1 which had been infected with BRSV 375 without animal passage were restricted to the lower respiratory tract and resemble the typical picture of BRSV infection. Two previous studies of experimental BRSV infections in which a combination of exposure routes (intranasal and intratracheal) was used led to similar results [17, 19]. The observation that areas of lobular consolidation were found in all lung lobes was in agreement with the observation of others [16]. However, the virus exposure did not produce extensive areas of lobular consolidation and such pneumonic proportions of the total pulmonary surface area as described in previous studies [16, 19]. Emphysema and edema have also been described in field reports and in experimental BRSV infections [16, 17, 19] but were not observed in the present investigation. On the other hand, severe bronchitis and tracheitis were common findings in all calves infected with the reisolated virus. This indicates that reisolated BRSV was capable
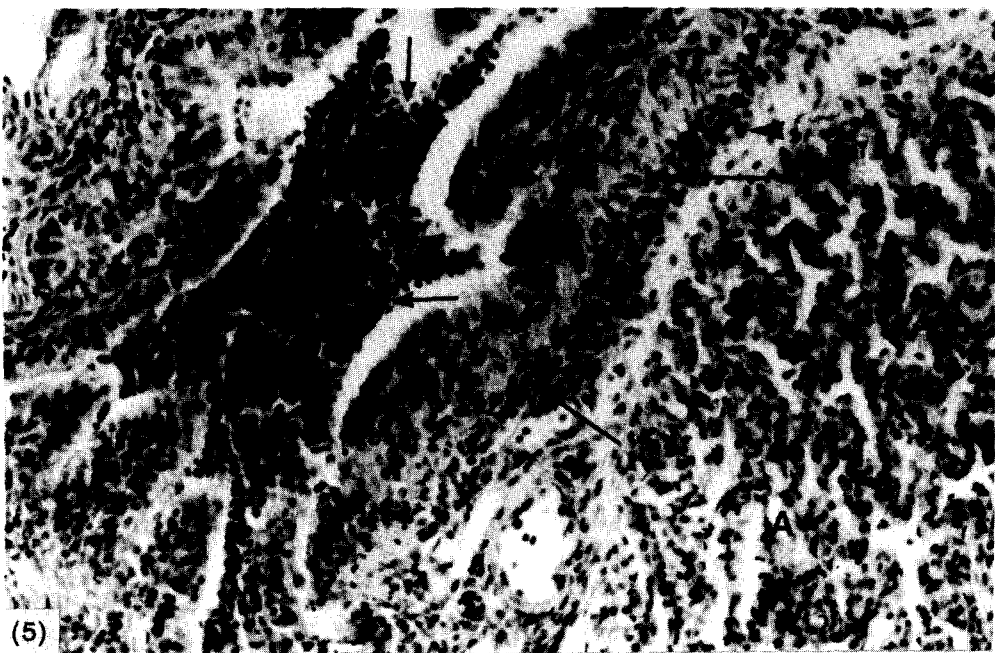
Fig. 4. Gross findings in the lungs consisted of irregularly distributed atelectasis (arrows). The atelectases were developed both in central and peripheral regions. Original magnification × 5.

Fig. 5. In the lumen of a collapsed bronchiolus a putrid exudate is visible (short arrows). In the bronchial wall lymphocytes (long arrows) and granulocytes can be found. The corresponding alveoli are collapsed (A). H & E. Original magnification × 400.
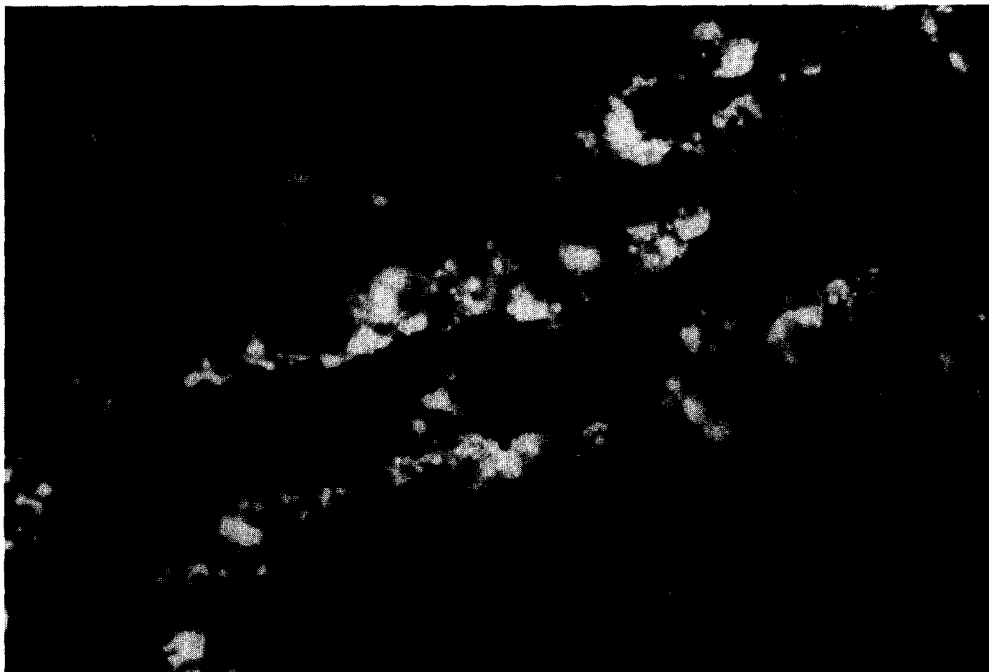
Fig. 6. Immunofluorescence in bronchiolar epithelium and in bronchiolar exudate in lung of calf No. 1 killed on day 7 after infection. BRSV proteins are recognized by IF with MAb 3C4 recognizing the protein P. Original magnification × 250.

of inducing tracheitis. The reason for this variation in pathological lesions seems to be the restoration of pathogenicity of the reisolated virus due to animal passage.

The presence of passive antibodies failed to prevent infection and disease caused by BRSV [19]. In the present study all calves possessed serum antibodies to BRSV prior to experimental infection. In comparison with results in a previous report [33], the frequency of virus recovery obtained after inoculation of BAL fluid specimens into BT cells was very high. These results lead to the conclusion that colostrum feeding did not influence the possibility of reisolating BRSV. The studies of Belknap *et al.* [16] have demonstrated similar results in calves. In contradiction to the findings of others [18], in our study an increase of antibody titres was not observed.

Viruses other than BRSV were not identified in any of the calves by viral isolation or HA testing. The need for microbiological examination of the respiratory tract was underlined by the isolation of bacteria. *Pasteurella* spp. were detected only in nasal swabs and not in the lower respiratory tract. This reflects that, in spite of the adverse effects caused by the aerosol infection, *Pasteurellas* were not able to spread to the lower respiratory tract. The isolation of *Mycoplasma bovirhinis* in nearly all nasal swabs of calves was not surprising. In a recent study the ubiquitous *M. bovirhinis* was isolated from nasal swabs of 23 out of 35 (66%) healthy calves [34]. Secondary infections with *M. bovirhinis* were observed in calves No. 1 (lung and trachea) and 5 (trachea), but electron microscopic and histological examinations did not reveal lesions typical of mycoplasmal pneumonia in our study.
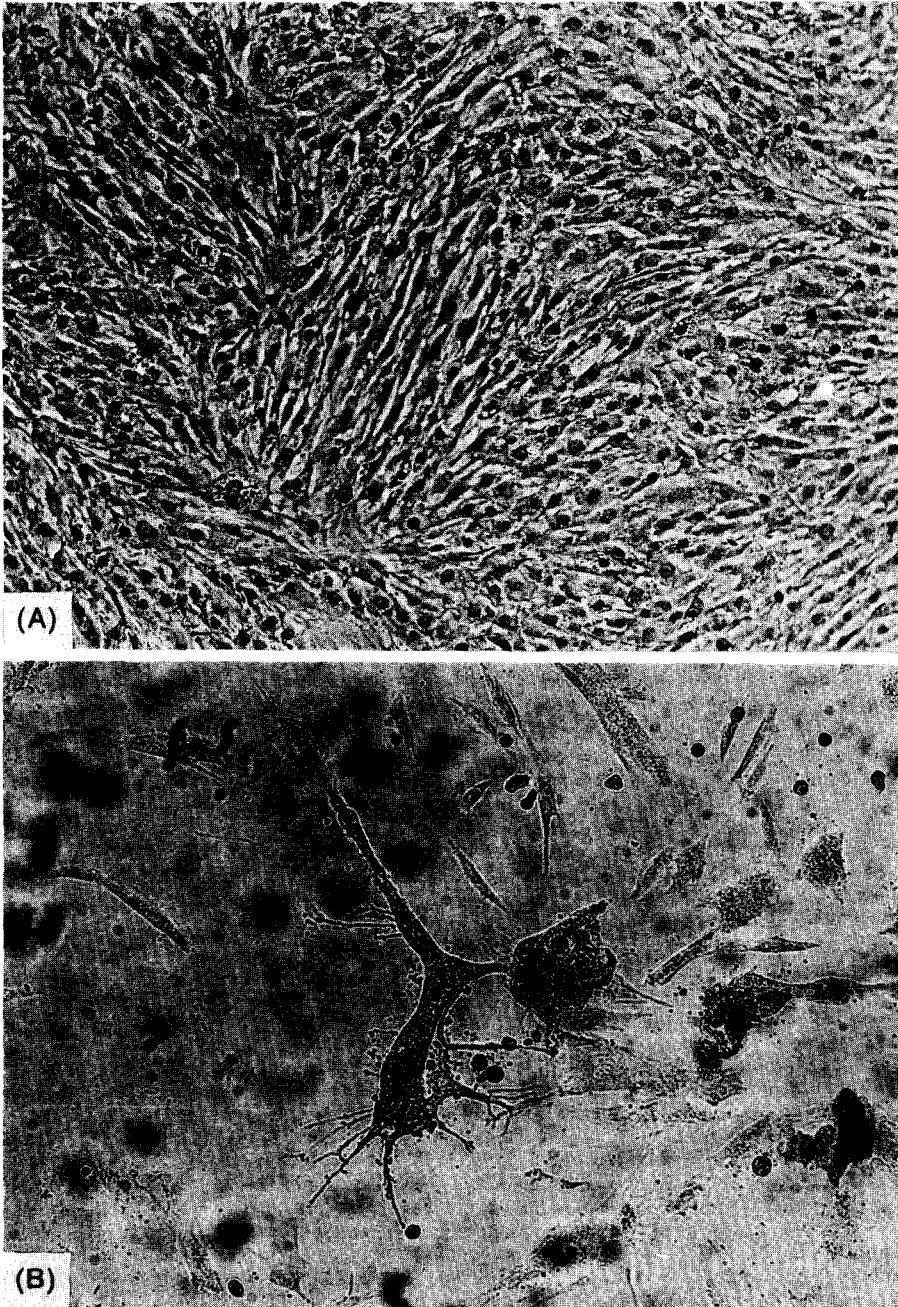
Fig. 7. BT-cells: (A) uninfected monolayer, (B) BRSV-infected cells 6 days after inoculation, focal
areas of cells are fused to form syncytia. Original magnification × 350.

The detection of BRSV by immunofluorescence test in bronchiolar epithelium and in a few alveolar cells, as well as in cellular debris within the lumina of the pulmonary airways, the isolation of the virus from the BAL fluids of all calves and the gross and histological findings indicate that the disease in the experimentally infected calves was produced by BRSV alone. Based on the obtained data, the described model of an artificial RSV infection is suitable for further investigations. Thus, this model can be used to clarify pathogenetic aspects of the RSV infection including changes in lung function and mechanisms of airway responsiveness. Furthermore, on the basis of this model new vaccines and immunization schemes can be studied in the future.

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