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Persistence of airway hyperresponsiveness and viral antigen following respiratory syncytial virus bronchiolitis in young guinea-pigs

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Persistence of airway hyperresponsiveness and viral antigen following respiratory syncytial virus bronchiolitis in young guinea-pigs. F. Riedel, B. Oberdieck, H-J. Streckert, S. Philippou, T. Krusat, W. Marek. ©ERS Journals Ltd 1997.

ABSTRACT: Respiratory syncytial virus (RSV) bronchiolitis in infancy is known to be followed by chronic respiratory symptoms and airway hyperresponsiveness in a subgroup of patients.

To further investigate the pathogenesis of RSV-induced chronic airway pathology, we infected young guinea-pigs at 4 weeks of age with RSV applied as an aerosol (n=30), and control guinea-pigs with virus-free culture medium (n=24). Infection was confirmed by positive antibody titre to RSV after 6 weeks, and by typical pathological changes of bronchiolitis after 1 week in six animals from each group. Airway hyperresponsiveness was measured weekly for 5 weeks by histamine challenge, using body-plethysmographic measurement of compressed air (CA). The provocative concentration of histamine producing significant airway obstruction (*i.e.* CA = 0.1 mL) ($PC_{0.1\ mL\ CA}$ in $mg \cdot mL^{-1}$) was calculated from dose-response curves. Six weeks postinfection, the lungs were investigated for the presence of inflammation and of viral antigen by immunofluorescence and immunohistochemistry using a rabbit hyperimmune serum and monoclonal antibodies.

Airway responsiveness was increased in the RSV group 1 week postinfection compared to the control group ($PC_{0.1\ mL\ CA}$ median 2.50 *vs* >10 $mg \cdot mL^{-1}$; $p<0.001$) and this persisted up to 5 weeks postinfection ($PC_{0.1\ mL\ CA}$ median 1.61 *vs* >10 $mg \cdot mL^{-1}$; $p<0.001$). During the same period, viral antigen persisted in the lungs of infected animals, although there was less inflammation at 6 weeks postinfection than at 1 week postinfection.

In guinea-pigs, respiratory syncytial virus infection of the airways causes persistent airway hyperresponsiveness over a period of at least 5 weeks. During this time, viral antigen, but not inflammation, remains detectable in the lungs and might be responsible for ongoing airway hyperresponsiveness.

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Respiratory syncytial virus (RSV) bronchiolitis in infancy has been reported to predispose children to the development of chronic respiratory symptoms, such as recurrent wheeze and asthma, in several epidemiological studies [1–5]. Furthermore, viral respiratory tract infections are frequently known to be followed by transient increase in airway reactivity, even in healthy humans [6, 7]. Following RSV infection in infancy, increased airway reactivity has been observed in a number of patients even after several years [8, 9]. It is still unknown from these observational studies whether it is the persistence of viral infection in the airway or other factors that are responsible for persistent airway hyperreactivity following RSV infection.

In the present study, a guinea-pig model of RSV-induced bronchiolitis was used to investigate the time course of virus-induced airway hyperresponsiveness, and, furthermore, to study the persistence of viral infection in the airways over a period of 6 weeks, using immunohistochemical techniques.

Material and methods

Animals

Dunkin-Hartley Pearlbright-White guinea-pigs (outbred, female, specific pathogen-free), weight 255±44 g (mean±SD), were obtained from Harlan-Winkelmann Co. (Borken, Germany) at 4 weeks of age, and housed under controlled conditions in an isolator with 12 h alternating light/dark cycles. Water and commercial food were allowed *ad libitum*. The animals were free from signs

of acute infection, and had not previously suffered from any known infection. Infected and noninfected groups were housed in a separate room, but maintained under identical conditions.

Protocol

A group of 24 guinea-pigs was infected with RSV aerosol on Day 1, 2 and 3 of the protocol. Beginning on Day 8 (*i.e.* the 5th day after the infection period), airway responsiveness to histamine was measured in the unsedated animals for a period of 5 weeks at weekly intervals, using unspecific histamine provocation tests in a two chamber body-plethysmograph.

Serum RSV antibodies were determined on Day 0 before infection and 6 weeks postinfection. At the end of the protocol (6 weeks postinfection), the animals were sacrificed and the lungs investigated histologically for the presence of inflammatory responses and of viral antigen.

An appropriate control group of 18 guinea-pigs was similarly treated, but was exposed only to an aerosol of supernatant from uninfected culture cells. Two other groups inhaled RSV aerosol (n=6) or supernatant from uninfected culture cells (n=6), but were sacrificed for histology at 1 day after the first airway challenge (Day 9, *i.e.* 6th day after the infection period).

Virus preparation

RSV (Long strain) was obtained from the American Type Culture Collection (ATCC) VR-26, and was propagated in human larynx carcinoma cell line (HEp-2) cells (Flow, Meckenheim, Germany). For virus production, subconfluent HEp-2 cells were infected with one focus forming unit (FFU) RSV per cell in Dulbecco's modified Eagle's medium (DMEM) supported with 1% foetal calf serum (FCS). Purification of the virus was performed as described previously by FERNIE *et al*. [10].

Inoculation of animals

The guinea-pigs were infected with RSV suspension supplied as an aerosol, for 45 min daily on three consecutive days. The aerosol was generated from 2×10^6 FFU RSV (diluted to a total volume of 5 mL with DMEM) by a compressor nebulizer (LC-type; Pari, Starnberg, Germany; mean mass diameter 3.1 µm, mean output 0.5 $mL\cdot min^{-1}$). The aerosol, which contained more than 60% particles smaller than 3 µm (according to the manufacturer) was administered to the guinea-pigs in an incubation box (20 L volume). Less than 10% of the initial RSV remained infectious after nebulization, as determined by virus titration.

The control groups were exposed to an aerosol of supernatant from uninfected HEp-2 cells instead of RSV suspension.

RSV antibody assay

The microneutralization assay applied was performed as described previously [11]. Briefly, 100 µL of the virus suspension, containing approximately 200 FFU, was mixed with 100 µL of the appropriate antibody dilution and incubated for 90 min at 37°C. One hundred microlitres of this suspension, together with 10^4 HEp-2 cells in 100 µL of medium, was seeded into the wells of a 96-well tissue culture microtitre plate. At 48 h postinfection the cells were fixed with ethanol and analysed for the presence of RSV proteins using a rabbit hyperimmune serum. Enzyme-labelling of bound antibodies was performed by means of a peroxidase conjugated anti-rabbit immunoglobulin G (IgG) antibody (Dako, Hamburg, Germany). 3-amino-9-ethyl-carbazole (Sigma, Taufkirchen, Germany) was used as substrate for enzyme reaction.

Virus isolation

HEp-2 cells, 2×10^5, were seeded onto the bottom of a 6-well cell culture plate (Costar, Badhoevedorp, The Netherlands). Lung tissue or cells from bronchoalveolar lavage (BAL) (approximately 10^6 cells) from two infected and two noninfected animals were applied to a transwell insert (24.5 mm diameter; pore size 0.3 µm; Costar).

Co-cultivation was performed in DMEM supplemented with 1% FCS in the presence of 0.2% gentamycin and 1% fungizone. After 48 h, the HEp-2 cells at the bottom of cell culture plates were fixed with ethanol. Immunochemical detection of RSV P-protein was performed with the monoclonal antibody 3C4 [12] and a peroxidase conjugated anti-mouse antibody (Dako, Hamburg, Germany). As mentioned previously, 3-amino-9-ethyl-carbazole (Sigma, Taufkirchen, Germany) was used as substrate.

Measurement of airway responsiveness

Pulmonary function measurements were performed in spontaneously breathing, nonsedated animals, using a two chamber body-plethysmograph, as described in detail previously [13]. Briefly, the animals were placed in the body chamber with the head in the respiratory chamber. A rubber cuff around the head separated the two chambers, in which gas conditions were kept constant (identical volume, 37°C, 100% humidity).

Pressure changes in the two compartments were recorded by sensitive pressure transducers (SP 2060; Svemor, Sweden; 0–6 Torr), and were volume-calibrated using a pump with defined volumes in the range of 1–4 mL. The pressure changes in both chambers were continuously recorded, stored and plotted against each other using an XY-recorder (Philips, Kassel, Germany).

With no resistance between body chamber (lung) and respiratory chamber (mouth), a straight-45° line would result. With airflow obstruction, the end-expiratory turning point deviated from the ideal-45° line, indicating compressed air (CA) in the lung, which could not be expired. The normal level of CA in spontaneously breathing healthy animals was found to be 0.025±0.012 mL (mean±SD). Significant airway obstruction was defined as CA of equal or ≥0.1 mL, representing >5 SD above the mean.

In order to measure airway responsiveness, histamine dihydrochloride (Serva, Heidelberg, Germany) prepared in phosphate-buffered saline (PBS), pH 7.4, and diluted to 2.5 mg·mL^{-1} with isotonic saline, was delivered into the respiratory chamber for 1 min using a nebulizer (IS-2, Pari; Starnberg, Germany), mean mass diameter 1.2 µm, mean output 0.32 mL·min^{-1}.

Prior to nebulization, baseline respiratory parameters were collected under steady state conditions. Breathing patterns were then recorded in 30 s intervals up to at least 5 min. The highest CA value recorded during this period was used for statistical analysis. After a recovery period of at least 2 h, the same animal was tested with doubled concentrations of histamine up to a maximum of 10 mg·mL^{-1} histamine, until a significant bronchoconstriction (CA >0.1 mL) occurred. The highest histamine concentration occasionally caused transient dyspnoea, but the animals recovered spontaneously.

The provocative concentration of histamine required to produce a CA equal to 0.1 mL ($PC_{0.1\ mL\ CA}$) was obtained manually from the concentration-response curve by logarithmic interpolation. If the animals did not react to the highest histamine concentration (*i.e.* 10 mg·mL^{-1}), a $PC_{0.1\ mL\ CA}$ value of >10 mg·mL^{-1} was used for calculation.

Histological and immunohistochemical examination

The animals were anaesthetized by intraperitoneal injection of 30 mg·kg^{-1} thiopental sodium (Trapanal; Byk Gulden, Konstanz, Germany) and sacrificed by exsanguination.

Afterwards, the chest was opened and the lung circulation was perfused *via* the right ventricle for approximately 3 min with isotonic warmed saline solution containing 0.2% heparin and 0.1% procaine (Hoechst, Frankfurt, Germany). Thereafter, the lungs were perfused for 20 min with 0.4% glutaraldehyde and 0.5% formaldehyde. The lungs were then removed and stored in 0.4% glutaraldehyde and 0.5% formaldehyde. Air-dried cryosections of lung tissue were fixed with 3.7% formalin in PBS for 10 min. Nonspecific binding, probably due to Fc receptors, was blocked with 0.5% human AB-plasma in PBS. Samples were processed for indirect immunofluorescence, as described above, using a hyperimmune serum raised against RSV [11]. For immune reactions, monoclonal antibodies against G- and P-proteins of the virus were also used. The specificity of the anti-RSV antibodies had been shown previously [12]. In two infected and two noninfected animals, a part of the lung was removed before fixation under sterile conditions and stored in PBS for virus isolation. In these animals, the trachea was opened before opening the chest, and the lungs lavaged twice with 5 mL of warmed PBS (BAL) using a small catheter inserted in the lower trachea. The recovery rate of BAL fluid, which was used for virus isolation, was about 80%.

For immunohistochemistry, enzyme-labelling was performed with an alkaline phosphatase-labelled antibody. Fast red/naphthol tablets (Sigma, Taufkirchen, Germany), containing levamisol for inhibition of endogenous alkaline phosphatase activity, were used to prepare the substrate solution.

Statistical analyses

Parameters of airway responsiveness in bronchial provocation tests ($PC_{0.1\ mL\ CA}$) are expressed as median with 95% confidence interval (95% CI), and were compared between the virus-infected group and the control group for each test day using the two-tailed Mann-Whitney U-test. A p-value of less than 0.05 was considered significant.

Results

Airway responsiveness

The results of the weekly airway challenge tests with histamine are presented in table 1.

Table 1. – Results of the weekly airway challenge, measuring the provocative concentration of histamine producing significant airway obstruction (*i.e.* CA=0.1 mL)

Postinfection week	$PC_{0.1\ mL\ CA}$ mg·mL^{-1} RSV (n=24)	Controls (n=18)	p-value
1	2.50 (1.34–5.00)	>10 (0.64–>10)	<0.001
2	3.54 (0.30–>10)	>10 (2.97–>10)	<0.001
3	3.10 (0.02–>10)	>10 (2.68–>10)	<0.001
4	4.44 (0.02–>10)	>10 (5.00–>10)	<0.001
5	1.61 (0.02–6.99)	>10 (1.24–>10)	<0.001

Values are presented as median, and range in parenthesis. RSV: respiratory syncytial virus; CA: compressed air.

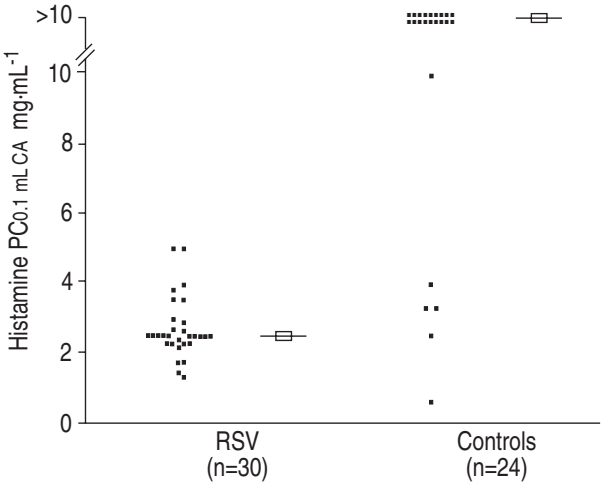

Fig. 1. – Airway responsiveness measured as provocative concentration of histamine leading to 0.1 mL compressed air ($PC_{0.1\ mL\ CA}$) 1 week postinfection, with median (——) and 95% confidence interval (▭) in RSV-infected guinea-pigs (n=30) and in controls (n=24). This figures includes the six animals in each group taken for histological examination 1 week postinfection. If the animal did not react with significant obstruction to the highest histamine concentration, a $PC_{0.1\ mL\ CA}$ value of >10 mg·mL^{-1} histamine was assumed for calculation. The difference in airway responsiveness between the two groups was highly significant (p<0.001). RSV: respiratory syncytial virus; CA: compressed air.

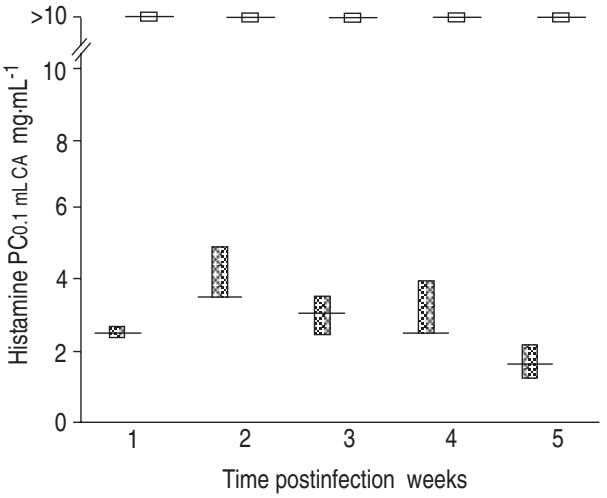

Fig. 2. – Airway responsiveness measured as $PC_{0.1\ mL\ CA}$ 1–5 weeks postinfection in RSV-infected animals (▭) (n=24) and controls (□) (n=18). The horizontal bars indicate the median value, and the boxes represent the 95% confidence interval. If the animal did not react with significant obstruction to the highest histamine concentration, a $PC_{0.1\ mL\ CA}$ value of >10 mg·mL^{-1} histamine was assumed for calculation. The two groups differed in $PC_{0.1\ mL\ CA}$ values throughout the period investigated ($p<0.001$). For definitions see legend to figure 1.

One week postinfection, the infected animals had increased airway responsiveness compared to the non-infected controls (fig. 1). Figure 1 includes the animals taken for histological examination on Day 9 postinfection (n=6 each for RSV and control group). In the animals in which weekly airway challenges were performed (RSV n=24, controls n=18), increased airway responsiveness after RSV infection persisted throughout the 5 week postinfection period of investigation (fig. 2).

Humoral immune response

Preimmune sera (Day 0) showed no detectable neutralizing activity against RSV. At 6 weeks postinfection, all virus-infected animals had RSV neutralizing antibodies of at least 1:100, whereas in the control group no seroconversion against RSV could be found. Therefore, virus infection was established in the infected animals, and cross-contamination of the control group could be excluded.

Histological examinations

RSV aerosol application induced the typical histological picture of bronchiolitis in this guinea-pig model 1 week postinfection in all infected animals. Atelectatic regions, distributed throughout the lung, were already visible by gross examination. Histologically, intraepithelial necrosis and infiltrations of neutrophil granulocytes were detected. The lumen of the bronchioli were filled with desquamated epithelial cells and granulocytes. In the bronchial wall, neutrophils and lymphocytes were visible (fig. 3). In central airways, mild inflammatory changes were to be seen.

At 6 weeks postinfection, signs of acute inflammation were rarely visible in the lungs of RSV aerosol-exposed guinea-pigs. A few poorly-ventilated regions (dystelectases) had developed throughout the lung (fig. 4). In control animals, histological examination revealed normal bronchial and lung tissue.

Detection of RSV proteins

Six weeks postinfection, the presence of viral proteins could be demonstrated (using immunofluorescence and

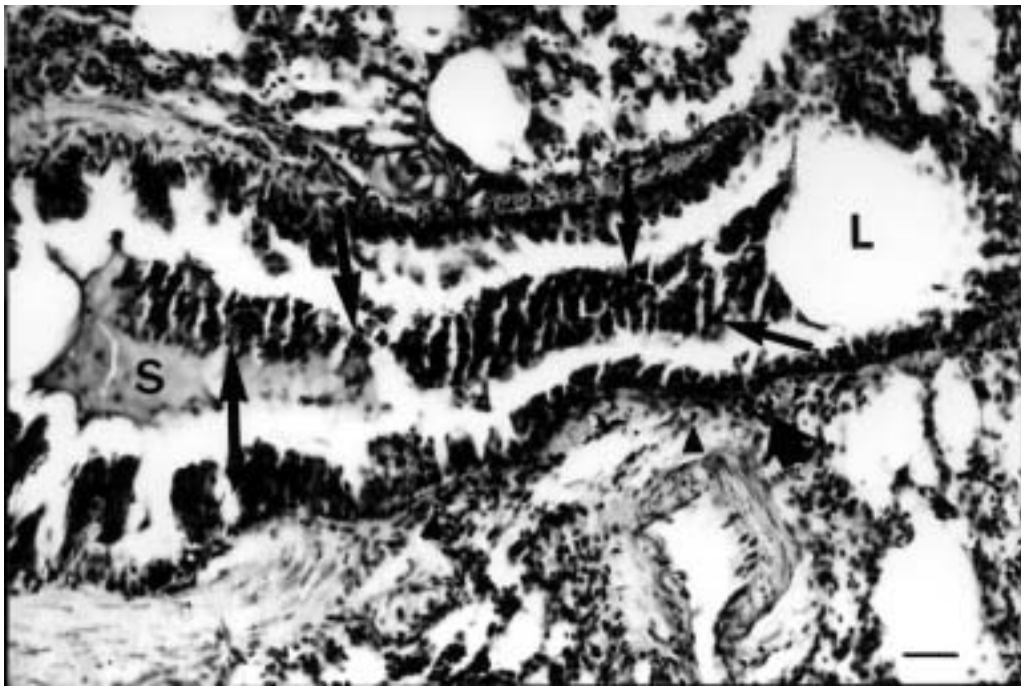

Fig. 3. – Bronchiolus of respiratory syncytial virus (RSV)-infected guinea-pig lung 1 week postinfection. The bronchiolus is filled with neutrophils (long arrows), desquamated epithelial cells (short arrows) and secretion (S). Neutrophilic granulocytes (arrow head) and lymphocytes (thick arrow) are also visible in the bronchial wall). (Internal scale bar=80 µm; haematoxyline and eosin staining).

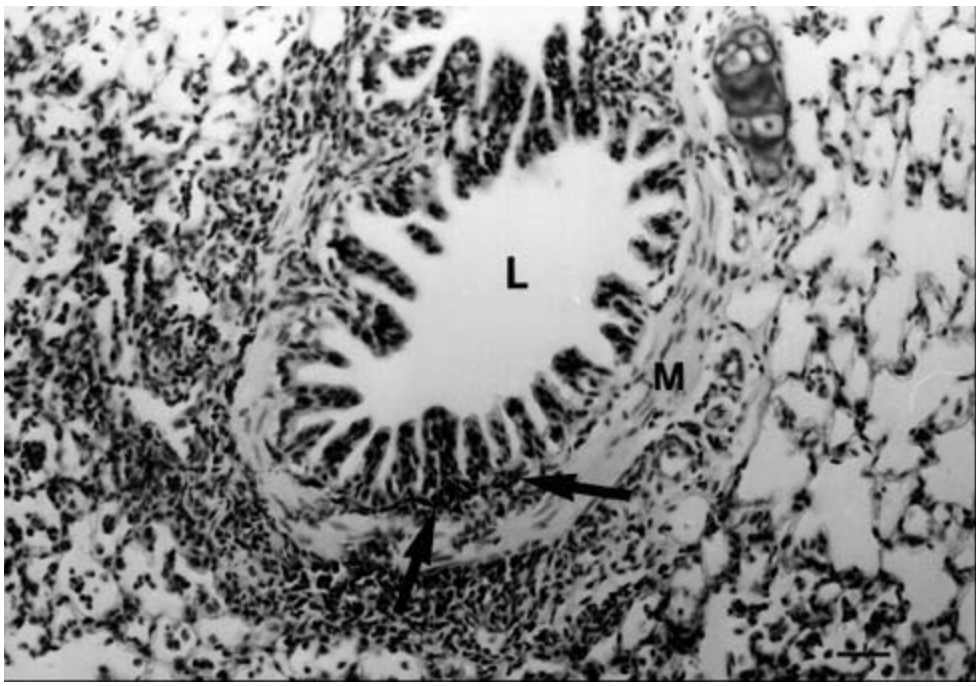

Fig. 4. – Bronchiolus of respiratory syncytial virus (RSV) infected guinea-pig lung 6 weeks postinfection. Only a few lymphocytes are visible (arrows) in the bronchial epithelium. L: bronchial lumen; M: airway smooth muscles. (Internal scale bar=80 µm; haematoxylin and eosin staining).

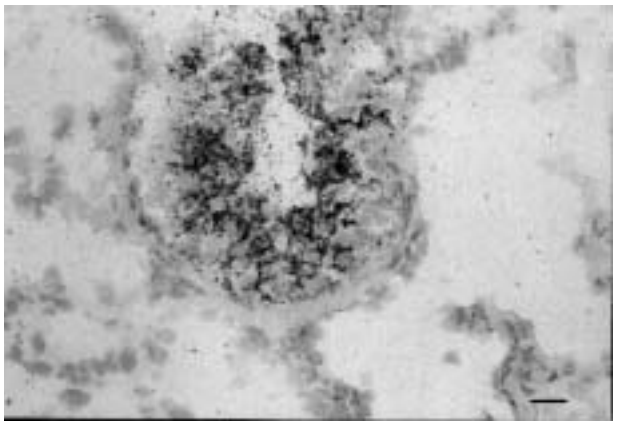

Fig. 5. – Cryosection of respiratory syncytial virus (RSV) infected guinea-pig lung 6 weeks postinfection. A positive immunohistochemical reaction using rabbit hyperimmune serum raised against purified RSV can be seen in the cytoplasm of bronchial epithelial cells. (Internal scale bar=160 µm; hematoxylin staining).

immunohistochemical techniques) mainly in the epithelium of bronchioli in the neighbourhood of regions showing pathological alterations (fig. 5). In all control animals immune reactions were negative.

Cryosections of infected lungs analysed by immunofluorescence (data not shown) and immunohistochemistry, performed with a hyperimmune serum raised against RSV and with monoclonal antibodies directed against G- and P-protein of the virus, revealed the presence of viral proteins in these sections. The proteins were detected mainly at the epithelium of small airways (fig. 5). Only intracytoplasmatic staining of infected cells was observed, as expected [12]. In the control group, no RSV-specific staining could be seen.

Replication of virus

RSV isolation from lung tissue and cells from BAL fluid was performed in two animals at 1 week after

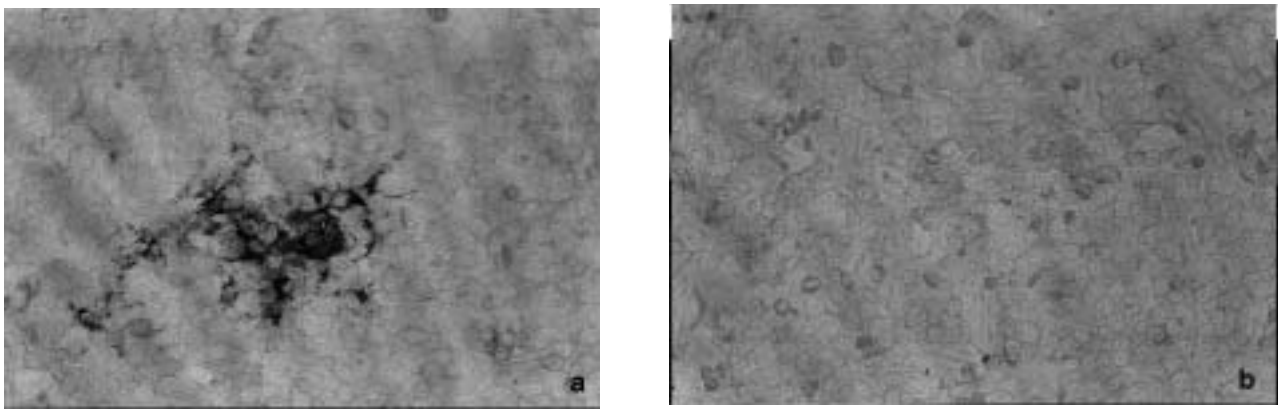

Fig. 6. – Reisolation of infectious respiratory syncytial virus (RSV) from lung tissue and bronchoalveolar lavage cells (BAL) from guinea-pig lung 1 week after experimental infection. a) Human larynx carcinoma cell line (HEp-2) cells co-cultivated with cells from investigated animals for 48 h were analysed for the presence of RSV P-protein. RSV P-protein was detected in the cytoplasm of HEp-2 cells. b) Control experiment performed with HEp-2 cells co-cultivated with material from uninfected control animals, no virus protein was detectable.(Internal scale bar = 25 µm).

experimental infection. At this time, infectious virus could be reisolated from lung tissue as well as from BAL cells of the two infected animals, whereas no virus isolation was possible from lung tissue or BAL cells of noninfected controls (fig. 6).

Discussion

In this guinea-pig model of RSV infection, airway hyperresponsiveness was seen 1 week postinfection. This observation was in concordance with previous studies using parainfluenza virus in guinea-pigs, in which the maximal bronchial hyperresponsiveness was found on day 6–8 postinfection [14]. However, in the present study, bronchial hyperresponsiveness persisted at least over the following 5 weeks when compared to the noninfected control group. Over the same period, virus protein persisted in the lungs of the infected animals, as shown by immunohistochemical techniques, despite the production of neutralizing antibodies.

For our infection protocol, we used young guinea-pigs at 4 weeks of age, since in human beings RSV infection usually takes place in the first 2 yrs of life [15], being equivalent to the first weeks of life in guinea-pigs. The resulting infection produced no overt clinical symptoms in this model, resembling subclinical infection in human beings. Only a minority of children with RSV infection develop acute lower airway disease requiring hospitalization [15].

RSV infection in this guinea-pig model was confirmed by culturing RSV from infected lungs as well as from cells obtained from BAL fluid of infected animals 1 week postinfection. Furthermore, in all animals tested, a humoral immune response with neutralizing antibodies at 6 weeks postinfection was found.

After application of RSV aerosol, the animals in the present study exhibited the classical histopathological features of acute bronchiolitis with corresponding atelectases 1 week postinfection, *i.e.* at a time point when a high inflammatory reaction of the airways to RSV infection had been described previously by HEGELE *et al.* [16] in their guinea-pig model. Six weeks postinfection, however, only very mild inflammatory changes of the bronchioli and few dystelectases were observed in the guinea-pigs in the present study, which was in agreement with the time course of histopathological changes following infection with parainfluenza virus type 3 (PIV-3) in guinea-pigs reported previously [14, 17].

FOLKERTS *et al.* [17] found epithelial damage in guinea-pigs after PIV-3 infection to be present from Day 2 up to Day 8, but not at Day 16, when *in vitro* tracheal reactivity was still increased in their model. Thus, these authors concluded that morphological changes were not directly associated with changes in airway hyperresponsiveness. In the present model, increased airway responsiveness was observed in comparison to the noninfected control group even at 5 weeks postinfection, at a time when inflammatory reactions were only minimal. In contrast to the histological picture, at this stage, viral proteins were still present in the epithelial cells of small airways and in dystelectatic or atelectatic regions of the lung, as proved by immunohistochemical staining.

In previous studies of lung parenchyma from experimental animals, RSV was usually isolated for a period not exceeding 2 weeks postinfection [18, 19]. However, persistence of viral antigens following RSV infection in the guinea-pig was already reported by HEGELE *et al.* [20]. In their animals infected intranasally with RSV, replicating virus was still present in 8 of 10 animals 2 weeks postinfection. Furthermore, genomic ribonucleic acid of RSV could be detected at 60 days postinfection in some animals [20].

Virus-induced airway hyperresponsiveness is not restricted to RSV or PIV-3 infection in animals, but has also been found in healthy humans following viral respiratory tract infection [6, 21], or experimental intranasal inoculation of rhinovirus [22] and influenza vaccine [23]. Persistent viral infection of the lung, as a sequel to acute bronchiolitis or other viral lower respiratory tract infection, might possibly also play a role in the pathogenesis of postbronchiolitic wheezing and chronic pulmonary symptoms following acute viral infection, including asthma. Therefore, RSV might chronically persist in the lungs as a possible stimulus for chronic airway inflammation [24].

In this guinea-pig model, only mild chronic airway inflammation was found 6 weeks after infection in comparison to 1 week postinfection. However, airway hyperresponsiveness and viral antigen remained unchanged over this period. Therefore, the presence of inflammatory cells in the interstitial tissue and airway lumen, which was assumed to be important for the development of airway hyperresponsiveness [25], might not be the only mechanism involved.

RSV might potentiate cytokine production and mediator release from resident cells in the epithelium and thereby enhance airway responsiveness. RSV has been shown to activate eosinophils *in vitro* to release various inflammatory mediators [26]. Furthermore, RSV was found to induce cytokine production by normal human bronchial epithelial cell lines [27].

In conclusion, we have demonstrated, using a guinea-pig model of RSV airway infection, that viral antigen persisted for at least 5 weeks after infection, despite adequate production of neutralizing antibodies, which were not able to clear the virus from the respiratory tract. Over the same time period, airway hyperresponsiveness was unchanged in infected animals, whereas inflammation of the airways was markedly diminished. Thus, antigen persistence, and not airway inflammation, was associated with persistence of airway hyperresponsiveness. Airway reactivity might possibly be potentiated by persistent virus-induced cytokine and mediator release, which in turn may be partly responsible for persistent airway hyperresponsiveness after respiratory syncytial virus bronchiolitis.

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