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Detection of respiratory syncytial virus (RSV) antigen in the lungs of guinea pigs 6 weeks after experimental infection and despite of the production of neutralizing antibodies

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Summary. Infections with respiratory syncytial virus (RSV) are characterized by frequently occuring reinfections and are regarded to be responsible for bronchial hyperreactivity. In this report we describe a small-animal model suited to study RSV-induced pathogenesis and immune response. Guinea pigs are infected by inhalation of an RSV-aerosol. Lungs of infected animals show signs of a bronchiolitis at 7 days after the initial infection. Although neutralizing serum antibodies are synthesized viral proteins are still detectable at 6 weeks post infection. Therefore, the presence of neutralizing antibodies is obviously not sufficient for rapid clearance of persistent RSV-proteins from the lungs of infected guinea pigs.

Introduction

Respiratory syncytial virus (RSV) is the major cause of severe lower respiratory tract disease in infancy and early childhood. Repeated infections frequently occur. Passively transferred maternal antibodies seem to protect infants during the first month of life [10]. On the other hand intravenously applicated immunoglobulin preparations containing RSV-neutralizing antibodies in high concentrations reduce the incidence and severity of lower respiratory tract disease [4]. It has been suggested that the close contact between blood and alveolar lumina may be responsible for protection of the lower airways [12]. Despite of this local protection of the lower airways, serum antibodies do not seem to provide resistance to subsequent infections [12]. Moreover, it was demonstrated that newborns and infants hospitalized on behalf of a RSV infection differ in their primary immune response from that observed in adults [3]. Surprisingly, these non-protecting antibodies seem to predispose infected children for severe course of disease. Although RSV may generally be isolated from infected children up to a maximum of 14 days [5, 6], an insufficient immune

response may result in virus shedding for several weeks [7, 11]. However, persistence of RSV is regarded to evoke bronchial hyperreactivity [1, 14]. Subject of this report are our studies concerning RSV-induced pathogenesis, immune response, and onset of viral replication in an experimental guinea pig model.

Materials and methods

Virus preparation

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

Inoculation of animals

4 weeks old Dunkin-Hartley guinea pigs were infected with RSV suspension supplied as an aerosol on 3 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 (Pari, Starnberg, Germany). The aerosol, which contained more than 60% particles smaller than 3 μm (according to the manufacturer's information), was administered to the guinea pigs in an incubation box (20 l volume; each group was simultaneously treated). Less than 10% of the initial RSV remained infectious after nebulization as determined by virus titration.

Three groups of guinea pigs were infected by an RSV aerosol: Group 1 ($n = 6$) was sacrificed 7 days post the initial infection (d. p.i.). Group 2 was studied 6 weeks post infection (w.p.i.) (initially $n = 6 - 1$ animal died during the experiment). Group 3 ($n = 6$) was used to monitor the kinetics of the immune response. Equal numbers of control animals inhaled supernatant from uninfected HEp-2 cells and were treated identically.

In addition 2 groups of guinea pigs ($n = 6$ each) were intranasally inoculated with 4×10^3, 4×10^4, or 4×10^5 FFU ($n = 2$ each) of an RSV suspension; 200 μl of the appropriate virus suspension was supplied via a teflon tube into each nostril: In group 4 pathological effects were examined 7 d.p.i.; group 5 was used to determine the immune response at 4 w.p.i.

Appropriate control groups to group 4 and 5 were similarly treated with supernatant from uninfected HEp-2 cells.

RSV-ELISA

ELISA was performed according to the described procedure [17] with purified RSV fixed to ELISA plates (Nunc, Wiesbaden, Germany). Enzyme labelling was done with a peroxidase conjugated rabbit anti-guinea pig antibody (Dako, Hamburg, Germany).

The cut-off for this test was defined as $Abs_{pos} \geqslant M\,(Abs_{neg}) + S_{neg}$ (Abs_{pos} represents the absorbance of a positive sample, $M(Abs_{neg})$ the arithmetic mean of adsorbances of negative samples, and S_{neg} the calculated standard deviation of the negative samples).

Micro-neutralization assay

The applied micro-neutralization assay was performed as described previously [3]. Briefly, 100 μl of the virus suspension containing approximately 200 focus-forming units

(FFU) were mixed with 100 µl of the appropriate antibody dilution and incubated for 90 min at 37 °C. 100 µl of this suspension together with 10^4 HEp-2 cells in 100 µl of medium were seeded into the wells of a 96 well tissue culture microtiter plate. At 48 h p.i. the cells were fixed with ethanol and analyzed 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 Ig antibody (Dako, Hamburg, Germany). 3-amino-9-ethylcarbazole (Sigma, Taufkirchen, Germany) was used as substrate for enzyme reaction.

Detection of viral antigen in cryosections

At the indicated time after infection animals were anaesthesized with 20–30 mg/kg body weight thiopental sodium (Trapanal, Byk Gulden, Konstanz, Germany) after premedication with 25 mg/kg body weight ketamine hydrochloride (Ketanest, Parke-Davis, Berlin, Germany) and 5 mg/kg body weight xylazaine (Rumpun, Bayer, Leverkusen, Germany). The chest was opened and via the right ventricle the lung circulation was perfused with isotonic saline solution containing 0.2% heparine and 0.1% procaine (Hoechst, Frankfurt, Germany).

Air-dried cryosections of lung tissue were fixed with 3.7% formaline in phosphate buffered saline (PBS) for 10 min. Non-specific binding probably due to Fc-receptors was blocked with 0.5% of human AB-plasma in PBS.

Samples were processed for indirect immunofluorescence as described [21]. The specificity of the anti-RSV antibodies has been shown previously [16, 21].

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

Results

Acute pathological effects

In our experiments pathological effects observed were dependent on the mode of application.

In the 6 guinea pigs intranasally inoculated with 4×10^3, 4×10^4, or 4×10^5 FFU of RSV no lung pathology was observed following necropsy on day 7 after the initial infection. Macroscopic and light microscopic evaluations did not show any significant differences between the lungs of infected or control animals.

In contrast, application of RSV as an aerosol was followed by clearly visible pathological effects significant for a bronchiolitis in the lungs of all infected animals on day 7 p.i.. Atelectatic regions distributed throughout the lung were already visible on gross examination. Light microscopic evaluations on paraffin embedded and frozen sections revealed regions with intraepithelial necrosis and infiltrations of neutrophil granulocytes (Fig. 1a). In the lumen of bronchioli desquamated epithelial cells and granulocytes were visible. By immunofluorescence the presence of viral proteins could be demonstrated mainly in the epithelium of bronchioli in the neighbourhood of regions showing pathological alterations (Fig. 2). In control animals the histologic examination and immunofluorescence were negative.

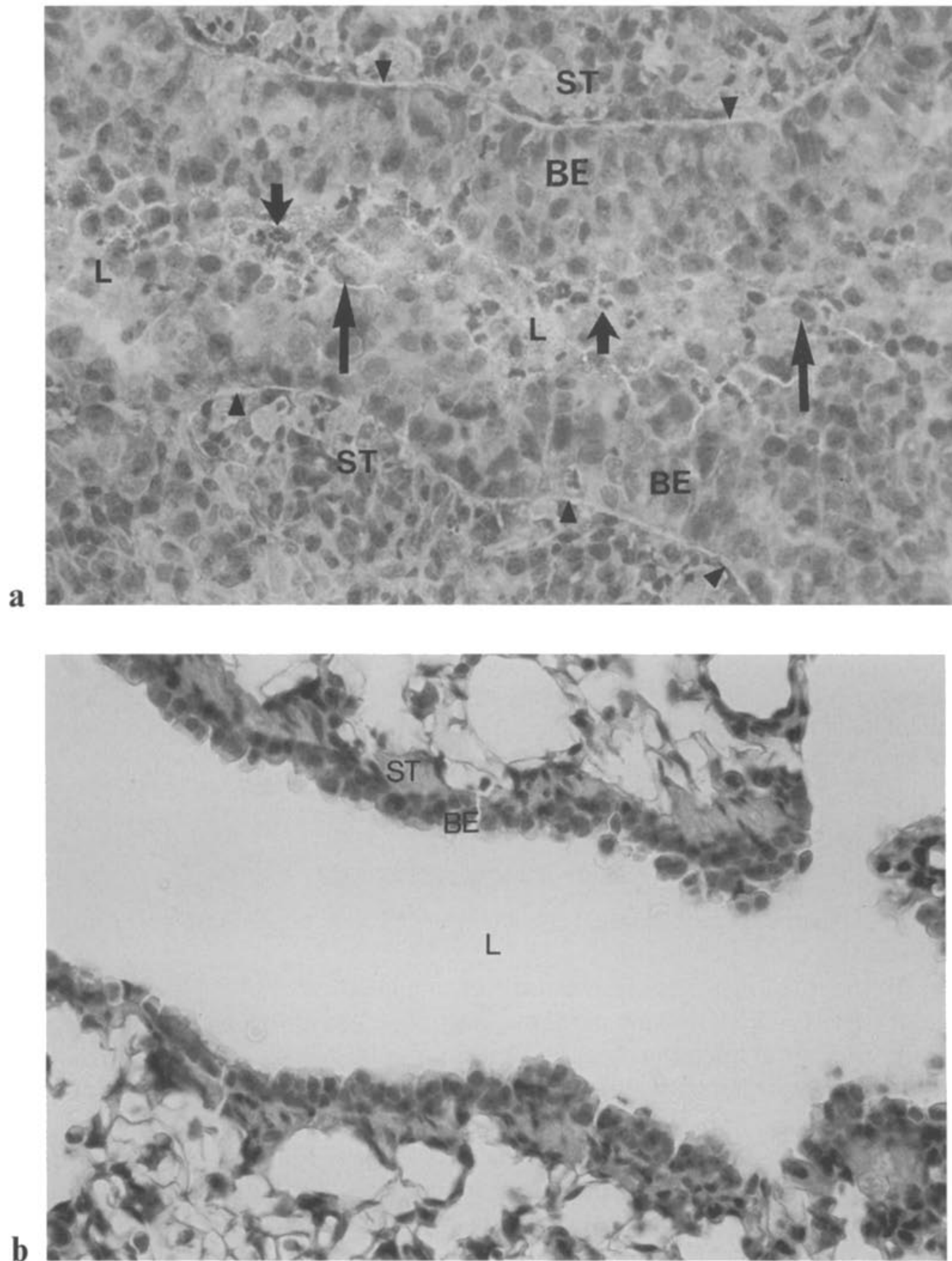
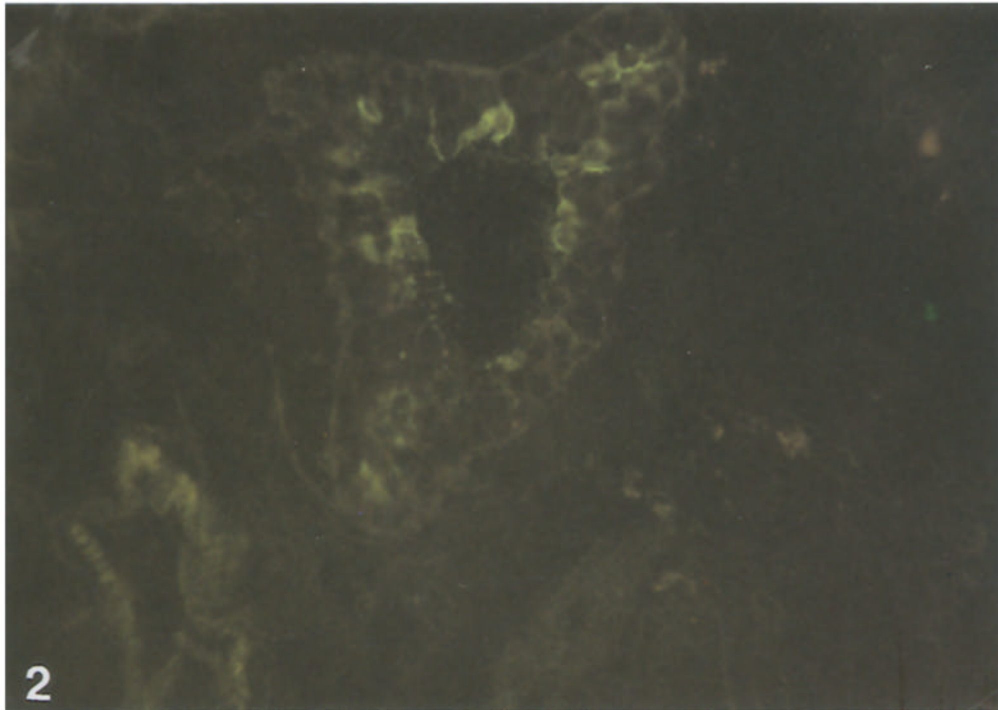


Fig. 1. Pathological effects in the lungs of a guinea pig that was experimentally infected with human RSV by inhalation (7 days p.i.). **a** Bronchioli in atelectatic regions of the lung are filled with putric exudate of necrotic epithelial cells and neutrophil granulocytes (HE-staining; original magnification: 1:500; *BE* bronchial epithelium, *ST* subepithelial tissue, *L* lumen; arrowheads: basement membrane, short arrows: neutrophil granulocytes, long arrows: desquamated epithelial cells). **b** Control to **a**; section from an uninfected animal (original magnification: $\times 250$)



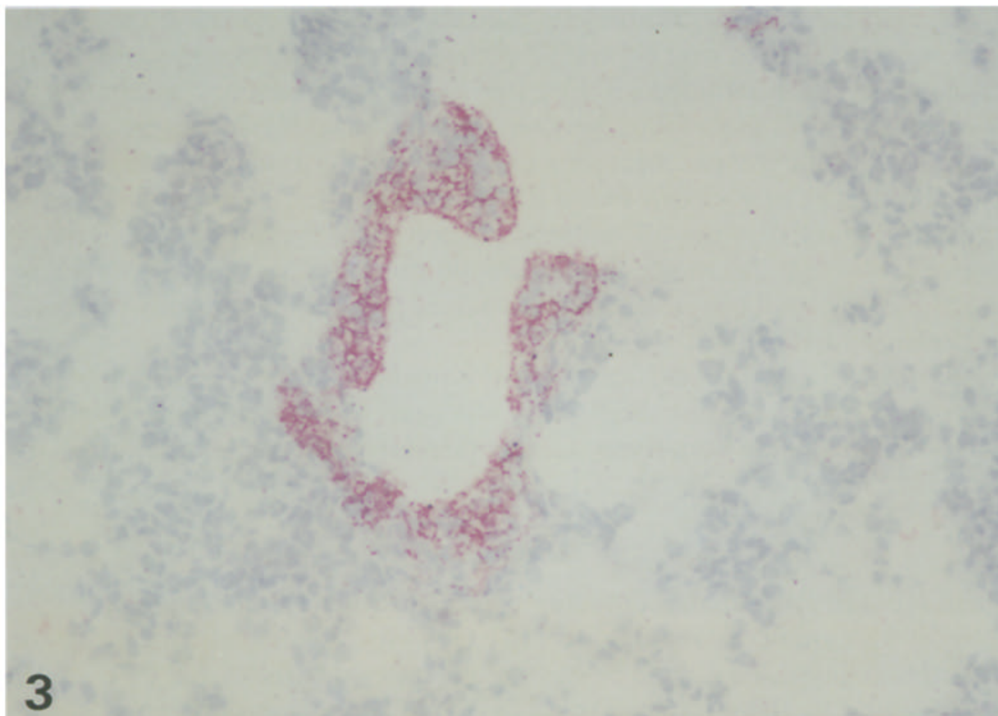


Fig. 2. RSV-proteins detected in cryosections of an infected guinea pig lung (7 d.p.i.; infected with an RSV-aerosol) by immunofluorescence with monoclonal antibody 3C4 (directed against P-protein) (original magnification: × 250). **Fig. 3.** Immunohistochemical detection of RSV proteins in a cryosection of a guinea pig infected by a RSV-aerosol at 6 w.p.i. RSV-proteins are detected by a rabbit-hyperimmune serum raised against purified RSV (original magnification: × 200)

Detection of RSV proteins at 6 weeks post infection

At 6 weeks p.i. signs of acute inflammation were rarely visible in the lungs of RSV-aerosol exposed guinea pigs. A few poorly ventilated regions (dystelectasis) could still be seen. Cryosections of infected lungs analyzed by immunofluorescence (data not shown) or immunohistochemistry either performed with an hyperimmuneserum raised against RSV or with monoclonal antibodies directed against G-protein or P-protein revealed the presence of viral proteins in these sections. The proteins are mainly detected at the epithelium of the small airways (Fig. 3) (sections from 5 infected guinea pigs were examined). Only intracytoplasmatic staining of infected cells was observed as expected [21]. In a control group no RSV-specific staining could be observed.

Humoral immune response

Sera taken from guinea pigs at 6 weeks p.i. were examined by ELISA and neutralization assay. All animals developed a significant response of ELISA-reacting antibodies (Fig. 4a) and moreover in RSV-neutralizing antibodies (Fig. 4b). Preimmune sera did not show any detectable neutralizing activity against RSV; the ELISA adsorbances for the preimmune sera were determined below the cut-off.

In a separate group of animals that inhaled RSV kinetics of the immune response were studied over 6 weeks: 2 of the animals had seroconverted already 2 w.p.i. (Fig. 5), 2 responded within 4 w.p.i., and the remaining 2 within 6 w.p.i.. No correlation could be found between titer and time of seroconversion.

In an additional experiment seroconversion after intranasal inoculation with 4×10^3, 4×10^4, or 4×10^5 FFU of RSV was monitored. Independent from the applied amount of virus all animals seroconverted within 4 w.p.i. (data not shown). Although this mode of infection did not induce lung pathology in our experiments, it shall be emphasized that guinea pigs may successfully be infected by the intranasal route.

Parallel to the infected animals all control animals were examined for a seroconversion against RSV. No humoral immune response could be monitored in the sera from these animals; therefore cross-contaminations can be excluded.

Discussion

Small laboratory animals were often regarded to be of limited interest for the study of RSV-induced bronchiolitis [15]. However, it could recently be demonstrated that guinea pigs could be successfully infected with 4×10^5 PFU of RSV (Long strain) by the intranasal route [9]. Mild bronchiolar inflammation was reported. Moreover, in contrast to cotton rat or mouse models, RSV could be isolated from 9 out of 10 guinea pigs on day 6 or 2 out of 10 animals 14 days post inoculation. The genomic RNA of RSV could be detected for 60 days after infection in some animals [8]. This indicates that human RSV is able to replicate in the lungs of infected guinea pigs.

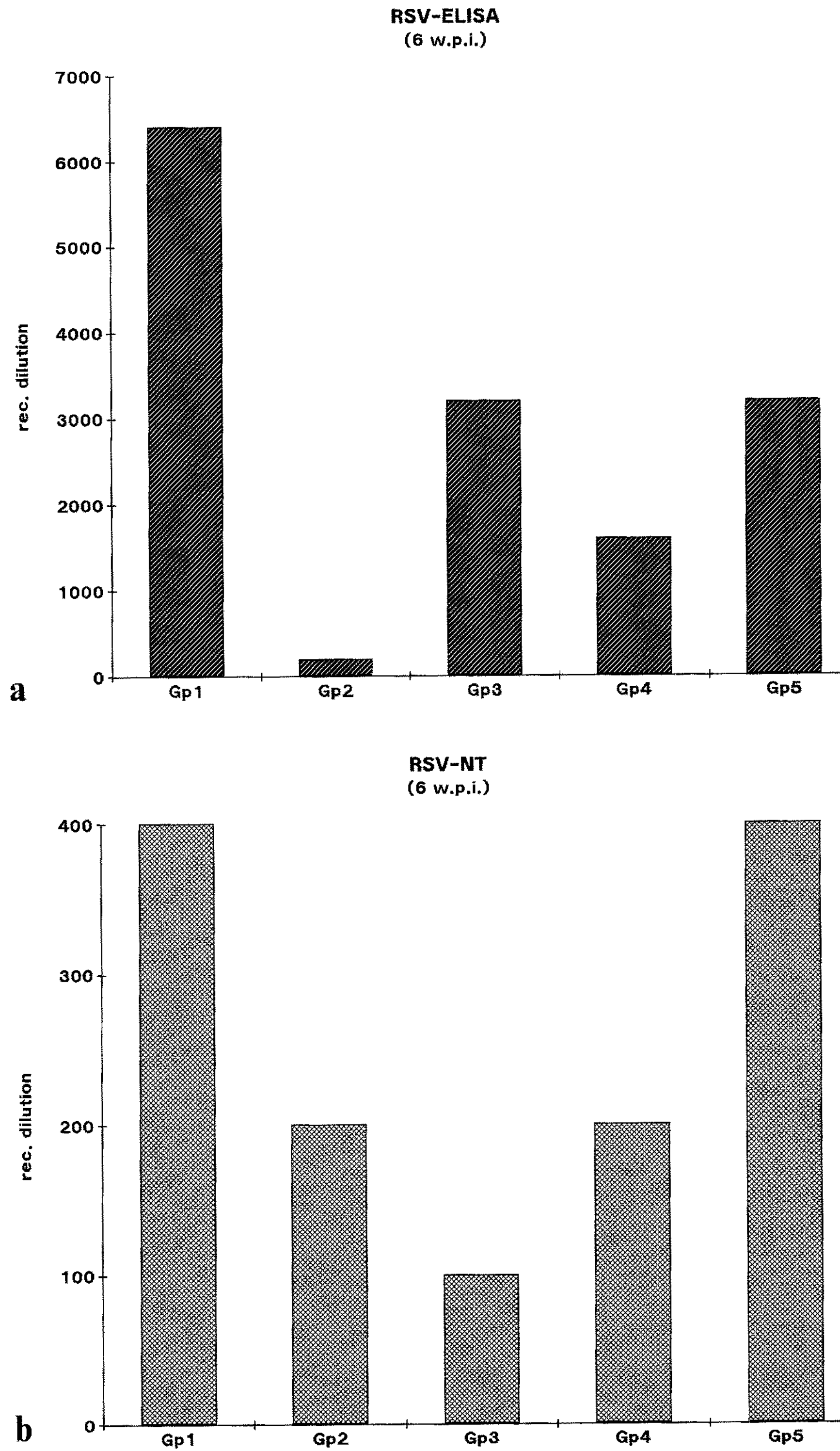


Fig. 4. Immune response of 5 guinea pigs with persisting RSV-proteins at 6 w.p.i. **a** ELISA reactive serum antibodies, **b** RSV-neutralizing activity of the antisera

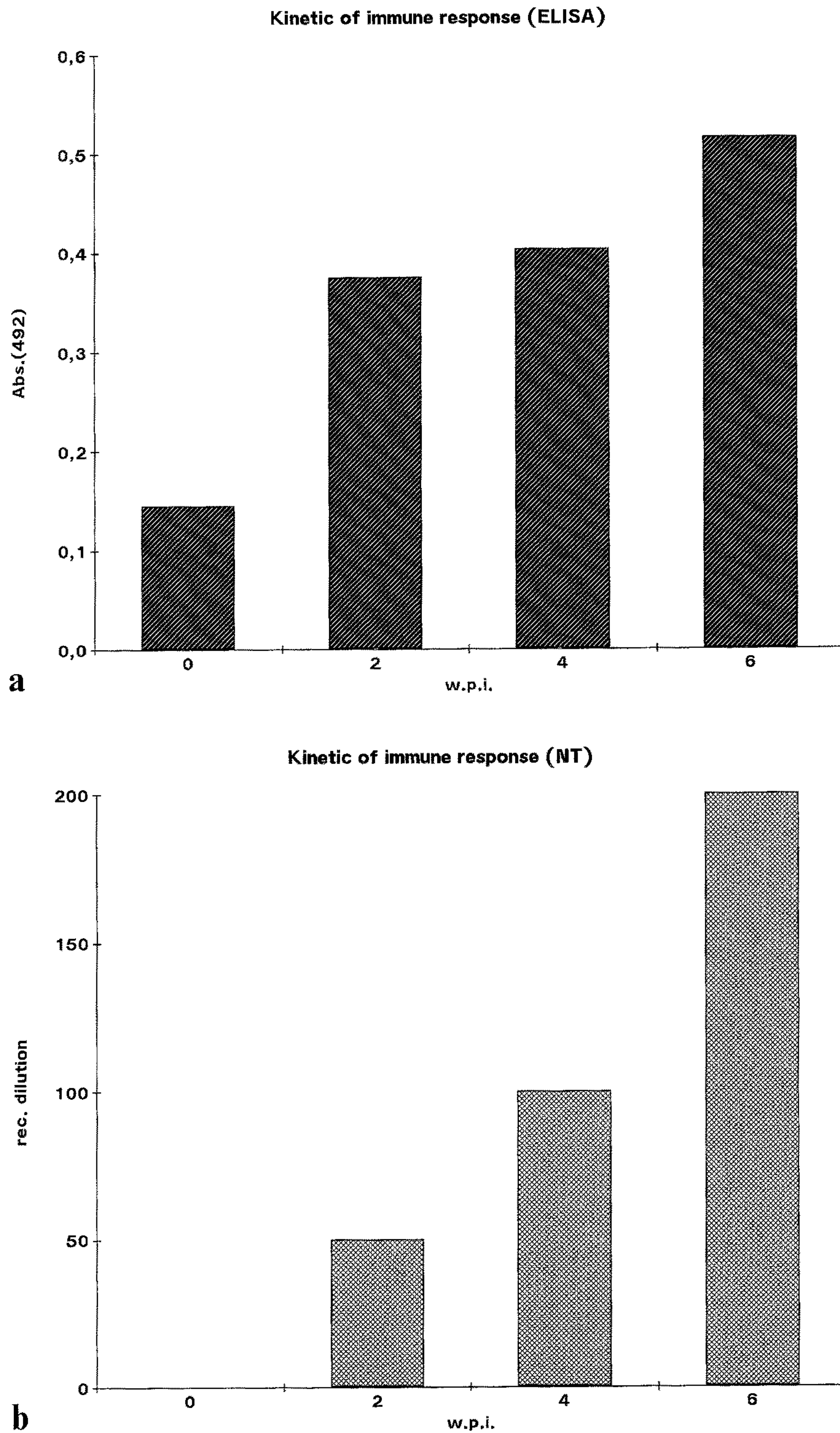


Fig. 5. Kinetics of seroconversion of a guinea pig after exposure to RSV-aerosol. **a** ELISA reactive serum antibodies, **b** RSV-neutralizing activity of the antiserum

In our experiments we were not able to observe significant acute phase lung pathology by intranasal application of 4×10^3 to 4×10^5 FFU of RSV, although a high titered humoral immune response was induced in a group of animals at 4 weeks p.i. Application of RSV as an aerosol with a mean particle diameter smaller than 5 μm resulted in significant signs of acute bronchiolitis distributed over the whole lung. The animals demonstrated an immediate humoral immune response against RSV. Moreover, neutralizing activity was associated with this primary immune response. Kinetic and quality of serum antibody synthesis was surprising, because it is in contrast to the situation in humans [3, 19] as well as in the experimental calf model [13], where the primary neutralizing immune response seems to be delayed. The reasons for this different situation in the guinea pig's immune response is still unknown, but is surely of interest for vaccine development. Recently published data [20] suggest that natural hosts or experimentally infected animals may differ in their immune response.

Despite of the induction of neutralizing serum antibodies RSV was obviously not cleared from the lungs of infected guinea pigs: mild lung pathology was still visible at 6 weeks p.i. and viral proteins could be detected at the epithelium of the small airways and in dystelectatic or atelectatic regions of the lung. Persistence of RSV-proteins is in agreement with the results of Hegele et al. [8] who detected RSV genomic material in infected animals.

In summary, it may be concluded from the presented data, that the application of RSV as an aerosol leads to enhanced lung pathology in the guinea pig. Therefore the guinea pig seems to be suited as an experimental model for RSV-induced bronchiolitis. However, the immune response differs for unknown reasons from that observed in humans or in calves. It is of some interest that the neutralizing humoral immune response monitored in guinea pigs fail to clear RSV-proteins from infected lungs. The demonstrated persistence of RSV-proteins in the epithelium is in agreement with the reported bronchial hyperreactivity frequently associated with RSV-infections.

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