

Parainfluenza-3-virus infection enhances allergic sensitization in the guinea-pig

F. RIEDEL, A. KRAUSE, W. SLENCZKA* and C. H. L. RIEGER

Department of Paediatrics, Ruhr-University Bochum, and *Department of Virology, University of Marburg, Germany

Summary

Background Viral respiratory tract infections have been previously considered to be associated with induction of allergic sensitization.

Objective and Methods In order to investigate this relationship in an animal model, guinea-pigs were inoculated intranasally with Parainfluenza-3-(PI-3) virus ($n = 16$) or virus-free culture medium (controls, $n = 12$), sensitized at day 4 with inhaled ovalbumin (OA) and challenged 3 weeks later with inhaled OA using specific bronchial provocation testing with body plethysmographic measurement of compressed air (CA). Furthermore, specific anti-OA-IgG1-antibodies in serum before challenge were determined by enzyme linked immunosorbent assay (ELISA). For investigation of airway epithelium permeability horseradish peroxidase (HRP) was inhaled at day 4 after inoculation by six animals, and HRP serum concentrations were determined by a direct ELISA 30 min after inhalation.

Results PI-3 infected animals were found to be significantly more sensitized to OA compared with controls, with higher CA values ($P < 0.001$) on specific bronchial provocation and with increased specific anti-OA-IgG1 titers. Serum-HRP concentrations were about 20 times higher in the infected animals compared with controls. PI-3 infected and sham-infected animals had comparable bronchial reactions on specific provocation with OA when sensitized systemically.

Conclusions We conclude that viral respiratory tract infection with PI-3 virus enhances inhalative allergic sensitization in the guinea-pig. Increased mucosal permeability to antigens may be an important pathophysiological mechanism.

Keywords: virus, sensitization, guinea-pig, ovalbumin

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Introduction

Viral respiratory tract infections have been known for many decades to trigger acute airway obstruction in asthmatics [1,2]. Even in healthy people they are followed by transient airway hyperreactivity [3]. Furthermore, acute bronchiolitis — a common viral disorder of the lower respiratory tract in infancy — is associated with long-term bronchial hyperreactivity, recurrent wheezing episodes [4] and the development of asthma. It is therefore of interest whether viral airway infections

can induce allergic sensitization of the respiratory tract.

Frick *et al.* [5] found an association between viral respiratory tract infections and the onset of allergic sensitization in children. In mice, infections with the influenza virus [6] and respiratory syncytial virus [7], followed by challenge with aerosolized antigen, enhanced the production of specific antibodies.

In order to investigate a possible association between experimental viral infection and induction of allergic sensitization, we exposed parainfluenza-3- (PI-3) virus infected guinea-pigs to aerosolized ovalbumin (OA) and monitored for specific bronchial sensitization using bronchial provocation tests with ovalbumin and measurements of specific anti-ovalbumin antibodies in serum.

Correspondence: Professor Dr F. Riedel, Universitäts-Kinderklinik, Alexandrinenstraße 5, D-44791 Bochum, Germany.

Materials and methods

Dunkin-Hartley pearlbright-white guinea-pigs (female, specific pathogen-free, weight 260–290 g) were purchased from Harlan-Winkelmann Company (Borken, Germany) at 4–5 weeks of age free of illness and kept on an ovalbumin (OA)-free diet throughout the experiment.

Viral culture

Bovine Parainfluenza (PI)-3 virus (Behring Company, Marburg, Germany, Op. 40387) was cultivated on HEp-2 monolayers in plastic flasks (Greiner Company, Frickenhausen, Germany) at 37°C in a humidified incubator containing 5% CO₂. Cell culture medium consisted of Dulbecco's minimal essential medium (Gibco Company, Eggenheim, Germany) supplemented with 5% fetal bovine serum (Gibco). Incubation took place for 11 days, the culture was renewed every other day. At the end of the incubation period, trypsin (2.5%, Gibco) was added to dissolve cell junctions and cell-plastic junctions.

The lysed cell suspension underwent centrifugation at $800 \times g$ at 4°C for 10 min, and the resulting supernatant was transferred to sterile tubes, shock frozen at –170°C and stored at –80°C. Supernatant from disrupted uninfected HEp-2 cells was obtained in a similar manner for inoculation in control animals.

Infectivity of the virus was shown by vacuolization and giant cell development of the cell layer. The final virus concentration in the medium was found to be 11×10^6 plaque forming units per mL.

Viral infection

A medium containing 200 µL of virus was inoculated in each nostril of 18 guinea-pigs with the head firmly supported, while in 12 control animals 200 µL of virus-free medium was inoculated in the same manner. Except sneezing on the first day of infection no symptoms were noted in infected or control animals.

The success of viral infection was monitored by measuring specific PI-3-antibodies in the serum of the animals on day 24 after infection using complement binding reactions in comparison to a positive anti-PI-3 serum (Virion Company, Würzburg, Germany).

Allergic sensitization

Four days after inoculation the animals were sensitized by inhalation of 0.5% ovalbumin (OA, grade VII, Sigma Chemicals Company, Deisenhofen, Germany), freshly prepared in isotonic saline. Inhalation took place in a

special device where the heads of the unsedated animals were fixed in a 50 mL inhalation chamber in front of a nebulizer outlet in order to optimize density of the inhaled allergen. OA solution was nebulized by air-driven nebulizer (Pari-Inhalierboy, Pari Company, Starnberg, Germany, output 0.3 mL/min, particle size 0.5–5 µm) over a period of 3 min. After 2 weeks the sensitizing OA inhalation was repeated in the same manner (booster inhalation).

Bronchial provocation

On day 25 after infection, sensitization to OA was investigated by specific bronchial provocation testing with OA. After measurement of baseline pulmonary function (see below) inhalation of nebulized OA (0.5% in isotonic saline) was carried out over 1 min using a 500 mL inhalation bag over the head of the animal and Pari nebulizer (see above). This was followed by repeated pulmonary function measurements over 11 min to monitor for bronchial obstruction.

Pulmonary function measurements were performed in spontaneously breathing non-sedated animals using a two-chamber body plethysmograph as described in detail elsewhere [8]. In brief, the animal was placed in the body chamber with the head in the respiratory chamber and a rubber cuff around its head separating the two chambers, in which gas conditions (identical volume, 37°C, 100% humidity) were kept constant. Pressure changes in the two compartments were recorded by sensitive pressure transducers (Svemor Company, Stockholm, Sweden. Type SP 2060, 0–6 Torr) and were volume-calibrated using a pump with defined volumes of 1–4 mL. The pressure changes in both chambers were continuously recorded, stored and plotted against each other using an XY recorder (Philips Company, Kassel, Germany).

Without any respiratory resistance between body chamber (lung) and respiratory chamber (mouth) a straight –45° line would result. With airflow obstruction the end-expiratory turning point deviated to the left from the ideal –45° line, indicating compressed air (CA) in the lung measured in millilitres, which could not be expired. This intrapulmonary volume has to be compressed on expiration before increased airway resistance is overcome. The normal range for CA in spontaneously breathing healthy animals was found to be 0.025 ± 0.012 mL ($\bar{x} \pm \text{SD}$). Significant airway obstruction was defined as CA of more than 0.10 mL, representing more than 5 SDs above the mean.

Two consecutive respiratory loops were recorded following antigen challenge every 2 min for a period of 11 min. The highest CA value recorded during this

period (usually 3–5 min after antigen inhalation) was used for statistical analysis. This form of pulmonary function testing has been shown to be more sensitive than measurements of airway resistance in spontaneously breathing guinea-pigs [8].

Antibody determination

For measurement of specific serum antibodies against OA, blood was obtained from all animals by puncture of the retro-orbital plexus before virus inoculation and on day 24 after infection, the day before the bronchial provocation testing.

Specific anti-OA IgG1 antibodies, the main reagenic antibodies in the guinea-pig [9], were determined by a direct ELISA as published previously [10]. Briefly, microtitre plates (Nunc Laboratories, Roskilde, Denmark) were coated with OA (grade VII, Sigma Comp.) at a concentration of 10 µg/mL and sealed with 0.1% gelatine. After addition of serial 1:5 dilutions of guinea-pig serum plates were incubated for 5 h. Plates were washed three times before the second antibody was added (rabbit anti-guinea-pig IgG₁ at a dilution of 1:2000). All preparations had been purchased from Miles Scientific Laboratories, Munich, Germany. Following incubation for 2 h at room temperature and three washes, alkaline-phosphatase-conjugated goat anti-rabbit IgG (Medac, Hamburg, Germany) at a concentration of 1:3000 was added and plates were incubated for 18 h at room temperature. Finally *p*-nitro-phenylphosphat (2 mg/mL) was added. The reaction was stopped after 100 min with 1 N NaOH and extinctions were measured at 405 nm in a multiscan photometer. Results were expressed as ELISA units (EU), defined as the reciprocal value of the serum dilution which gave an optical density of 1.0. In order to assure reproducibility, a known serum pool of guinea-pig anti-OA was run with each test as a standard. The level of detection was 20 EU.

Bronchial eosinophils (EOS)

As a marker for late allergic reaction after OA provocation, eosinophils were counted as percentage of cells in broncho-alveolar lavage (BAL) fluid obtained 24 h after specific bronchial provocation. For BAL procedure the trachea was prepared under barbiturate anaesthesia, a small tube was passed through an incision of the anterior tracheal wall and fixed with a clamp. Lavage of the whole bronchoalveolar system was performed twice with 5 mL of warmed isotonic saline, the recovery rate being 80% in the mean. There were no differences in the recovery volumes between infected and control animals. The BAL

fluid was centrifuged at $200 \times g$ at 4°C for 10 min and the cell pellet resuspended with 1 mL of phosphate buffered saline. Total cells and EOS were counted in Neubauer chamber using Türk's and Dunker's solution.

Unspecific airway responsiveness

For measuring unspecific airway responsiveness histamine dihydrochloride (Sigma) was delivered for 1 min to the head of the animal using a compressor nebulizer (IS-2, Pari Company, Starnberg, Germany; mean mass diameter 1.2 µm, mean output 0.32 mL/min), while the animal was seated already in the bodyplethysmograph. Directly after nebulization breathing patterns were recorded at 30 s intervals up to 5 min. The highest CA-value recorded during this period was used for statistical analysis. After a rest period of at least 2 h the animal was tested with the doubling concentration of histamine until significant airway obstruction ($CA > 0.1$ mL) occurred. The histamine concentration required to cause a CA equal to 0.1 mL ($PC_{0.1 \text{ mL CA}}$) was calculated from the concentration-response-curve by logarithmic interpolation.

Mucosal permeability

In order to measure permeability changes of the respiratory epithelium due to PI-3-virus infection, a group of six animals inhaled horseradish peroxidase (HRP) 0.5% (Sigma, endotoxin-free) freshly prepared in isotonic saline, using the same equipment and technique as described above for inhalative sensitization with OA. Again, HRP-inhalation (over 3 min in inhalation chamber as described above for OA) took place on day 4 after inoculation with PI-3-virus ($n = 3$) or with virus-free medium ($n = 3$). For measurement of HRP-permeability of airway epithelium serum was obtained 30 min after the end of the inhalation and the concentration of HRP in the serum was measured by a sensitive ELISA.

Briefly, microtitre plates (Nunc Laboratories, Denmark) were coated with Goat-Anti-HRP 1:1000 (Sigma) in Na_2CO_3 -buffer, incubated over night and washed with Tween 20 (Sigma) in isotonic saline. After addition of serial 1:2 dilutions of guinea-pig serum plates were incubated for 1 h at 37°C. After washing TMB (Behring) 1:10 diluted with buffer was added and the plates incubated for 3 h under light exclusion at room temperature. The reaction was stopped with 0.5 N H_2SO_4 and extinctions measured at 450 nm in a multi-scan photometer. Results were expressed as EU (see above). For standardization HRP (Sigma) was dissolved in buffer in 16 dilutions, beginning with 1 mg/mL, and measured accordingly instead of guinea-pig serum.

Systemic sensitization

In order to differentiate between viral infection induced change of mucosal permeability and influence on the immune response to antigens caused by the viral infection, 12 animals were sensitized with systemic OA application (50 mg OA grade VII in 1.0 mL isotonic saline i.c.) 4 and 18 days after intranasal PIV-3 infection ($n = 6$) or application of virus free supernatant ($n = 6$). One week after the last sensitization inhalative OA challenge was performed as described above.

Statistical analyses

Lung function parameter (CA), specific IgG1 (EU) values, eosinophilia in BAL (% total cells) and PC_{0.1 mL CA} values of histamine were compared between PI-3-virus- and control group using the Mann-Whitney-*U*-test for unpaired samples. Positive reactions on specific IgG₁-ELISA for OA were compared between viral and control groups using Fisher's exact test.

Results

All animals exposed to PI-3-virus ($n = 16$) showed an antibody response to the virus on day 24 after infection (complement binding reaction more than 1:32; eight times 1:64, five times 1:128 and three times 1:256), while control animals ($n = 12$) had no detectable antibodies to PI-3-virus. Weight gain was not different in both groups.

On specific bronchial provocation testing with OA the infected animals reacted with a significantly higher degree of bronchial obstruction compared with control animals (CA 0.42 [0.20–0.45] vs 0.08 [0–0.11] mL, median with 95% confidence intervall, $P < 0.001$, see Fig. 1). Two animals in the infected group died due to severe airway obstruction on specific OA provocation.

Specific anti-OA-antibody of the IgG₁ subclass (major anaphylactic antibody of the guinea-pig) were not detectable (< 20 EU) in any of the animals at the beginning of the experiment. At the end of the sensitizing period (day 24 after inoculation) none of the control animals again had detectable antibodies (< 20 EU), whereas seven of 16 virus-infected animals had detectable circulating specific IgG₁ antibodies. This difference was statistically significant using Fisher's exact test ($P < 0.01$). The absolute figures for anti-OA-IgG1 in EU are presented in Fig. 2, the difference of IgG1 titre levels between controls and virus infected animals is of only marginal significance ($P = 0.051$).

As evidence of a late allergic reaction, we compared EOS as proportion of total cells in bronchoalveolar

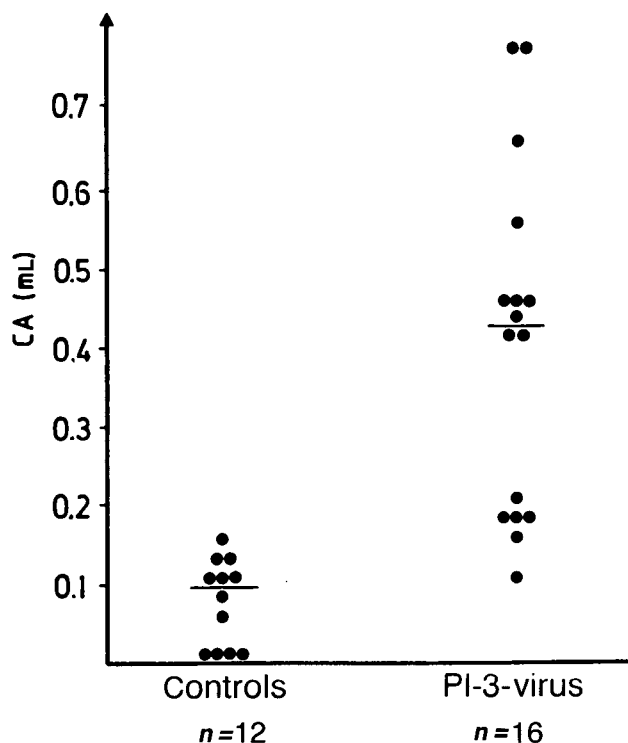


Fig. 1. Bronchial reaction on specific provocation with OA, bodyplethysmographic measurement of Compressed Air (CA) in mL as parameter for airway obstruction; —, median, $P < 0.001$.

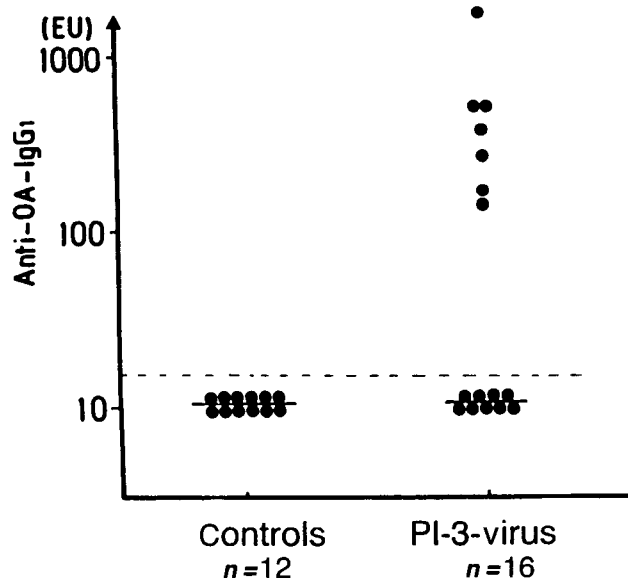


Fig. 2. Specific anti-OA-IgG1-antibodies in ELISA Units (EU) in the serum before provocation, logarithmic scale; —, level of detection (20 EU), $P = 0.051$.

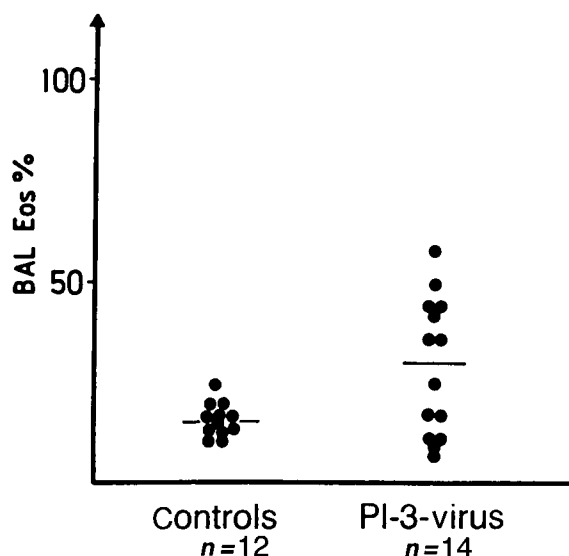


Fig. 3. Eosinophils (EOS) in percentage of total cells in BAL 24 h after provocation with OA as parameter for late allergic reaction (two animals in the virus group died on provocation, their data on BAL are not available); —, median.

lavage fluid 24 h after specific bronchial provocation between controls and virus infected animals. They were found not to be different between the two groups, although there was a trend towards higher values in the infected group (30 [17–43] vs 15 [13–17], $P = 0.057$, see Fig. 3). Unfortunately two animals died during bronchial provocation with severe bronchial reactions and could not be analysed for EOS in the BAL 24 h after provocation.

In the Table the parameters CA, Anti-OA-IgG₁ and EOS of the PIV-3 infected animals are listed according to the reaction on bronchial provocation with OA (a: mild reaction, b: medium reaction, c: severe reaction). Increased Anti-OA-antibodies in serum were only found in the subgroup with severe reactions on OA challenge (c). Furthermore, in the subgroup with no or mild OA reactions (a) a normal EOS count in the bronchoalveolar lavage fluid was observed, while EOS were increased (>20%) in the subgroups with medium and severe reactions to OA inhalation (b and c).

In the group for investigation of airway responsiveness, unspecific airway hyperresponsiveness to histamine was present in the six animals infected with PI-3 compared with the six controls with inoculation of virus-free supernatant from uninfected cells ($PC_{0.1 \text{ mL CA}} 3.3 [1.9–4.3]$ vs $4.2 [4.2–8.4]$ mg/mL histamine, median with 95% confidence interval, $P < 0.01$). As histamine challenge was performed 4 days after inoculation, we might assume, that in the PI-3 infected OA sensitized group

Table 1. Sensitization parameter of PI-3 infected animals listed according to severity of bronchial reactions on specific provocation with OA

	CA (mL)	Anti-OA IgG1 (EU)	Eos (%)
a	0.10	< 20	6.8
	0.15	< 20	10.6
	0.165	< 20	17.9
	0.175	< 20	9.2
	0.175	< 20	17.2
	0.20	< 20	11.8
b	0.40	< 20	25.1
	0.40	< 20	35.9
	0.40	< 20	43.3
c	0.45	139	42.7
	0.45	166	57.3
	0.45	1800	43.9
	0.55	518	35.0
	0.65	362	48.2
	1.00	269	+
	1.00	518	+

a: mild reaction, b: medium reaction, c: severe reaction. CA (mL): compressed air. Anti-OA-IgG₁ (EU): Anti-OA-Antibody of IgG₁ subclass in ELISA units. EOS (%). Eosinophils in percentage of cells in bronchoalveolar lavage fluid. +. Died on specific provocation before lavage.

unspecific airway hyperresponsiveness was present at the day of first sensitization with OA.

The permeability of the respiratory epithelium for HRP was compared between three animals with viral infection and three control animals following HRP inhalation 4 days after inoculation. HRP concentration in serum as a parameter for permeability was high in the infected animals and very low in the control animals (16.4, 20.4 and 22.3 ng/mL vs 0.4, 0.9 and 1.3 ng/mL [no overlap]).

After systemic sensitization with ic OA bronchial reaction on bronchial provocation with inhaled OA was not different inbetween PI-3 infected and sham-infected animals (0.35 [0–0.45] mL CA vs 0.15 [0–0.30] mL CA, median with 95% confidence interval, $P > 0.05$).

Therefore, PI-3 infection of the airways had no influence on the systemic sensitization by ic antigen application, but significantly enhanced sensitization to inhaled antigen.

Discussion

In 1979 Frick *et al.* [5] reported on an association between viral respiratory tract infection and the onset of allergic sensitization in young children. They

performed regular immunological studies over a period of 4 years in 13 children with a strong atopic disposition. In all 11 children, who developed symptomatic allergic disease, the onset of allergic manifestations was preceded by a proven viral respiratory tract infection.

The same authors studied the effect of live virus vaccine on IgE-response to parenterally administered allergens in dogs [11] and found the vaccine to promote primary IgE response to the allergen compared with non-vaccinated littermates.

Studies in mice revealed similar results: Sacamoto *et al.* [6] infected C3H-mice with influenza A virus before challenge with aerosolized OA as allergen and found an enhanced capacity of the inhaled allergen to trigger specific anti-ovalbumin-IgE production in these infected animals, while no specific antibody was detectable without the preceding viral infection.

Leibowitz *et al.* [7] studied RSV infection and inhaled OA in BALB-C mice and again found increased specific IgE production to OA in infected vs non-infected mice.

In this paper we present data on PI-3-virus infection and allergic sensitization in the guinea-pig. The concentration of the parainfluenza virus inoculated in the nose was low, thus overt clinical disease as shallow breathing or coughing was not observed. However, infection with the virus was demonstrated by a significant increase in anti-PI-3-virus antibodies in all infected animals, while in the control animals no detectable antibody to PI-3-virus was found.

Enhanced sensitization to inhaled OA as allergen was shown in the infected animals compared with non-infected animals by specific bronchial provocation tests using sensitive body plethysmographic measurements. Compressed air as sensitive marker for airway obstruction in the guinea-pig [8] was significantly higher on specific bronchial provocation with OA in the infected animals compared with the controls (Fig. 1).

It is unlikely that the bronchial reactions on specific bronchial provocation test with OA was due to unspecific effects of the preceding viral infection instead of specific sensitization, as the specific provocation took place 25 days after the infection.

Furthermore, specific anti-OA-antibodies of the IgG₁ subclass, the major homocytotropic antibody in the guinea-pig [9], were higher in the serum of the infected animals compared with the non-infected animals, measured at the end of the sensitizing process, again indicating enhancement of sensitization by the preceding viral infection (Fig. 2).

Our pulmonary function protocol was not designed to monitor for late allergic reactions after allergen provocation. Therefore, we measured the EOS response in the BAL fluid 24 h after specific provocation, as high

eosinophilic counts have been found in late asthmatic reactions in the guinea-pig [12].

In the virus-infected group the percentage of eosinophils on total cells in the BAL was not significantly higher than in the control group (Fig. 3).

However, two animals from the viral group died during the specific provocation due to severe airway obstruction, and BAL fluid could therefore not be analysed 24 h later. Thus, we do not know the eosinophilic count in the BAL in these two animals.

Increased permeability of the airway epithelium for inhaled allergens could be the consequence of virus induced inflammation and damage of the airway epithelium and might be followed by enhanced sensitization. Richardson *et al.* [13] described in a chicken model increased tracheal permeability after viral infections.

We therefore studied the permeability of inhaled HRP, a molecule with similar molecular weight (about 40 000 Da) compared with OA, as a sensitive direct HRP ELISA was established in our laboratory.

HRP was inhaled on day 4 after infection, the same day on which OA inhalation for sensitization was performed. HRP uptake through the lung was monitored by determination of HRP serum concentration using a sensitive ELISA. Increased HRP concentrations were found in all PI-3-virus infected animals compared with non-infected controls, indicating increased permeability to inhaled HRP due to the preceding viral infection, although the number of studied animals was small.

The pathophysiology of increased permeability has not been investigated in this study as histological examinations were not performed. However, epithelial disruptions and ultrastructural changes after parainfluenza infections have been shown by Castleman in rats [14].

In a parallel experiment airway responsiveness was measured 4 days after PI-3 infection and found to be increased compared with a control group of uninfected animals. As OA sensitization took place on this day in the sensitized animals, this fact further strengthens the hypothesis, that viral induced mucosal changes play an important role in enhanced sensitization after PI-3 infection in this animal model.

Besides increased allergen permeability due to virus-induced epithelial damage and inflammation, an alteration of immune response to inhaled allergens could be discussed as the consequence of viral infections.

Holt *et al.* [15,16] showed that repeated exposure of mice and rats to aerosolized allergens induces development of inhalative tolerance to the allergens by down regulation of the subsequent immunological reaction via a high level of specific suppressor T-cell activity. Influenza A-virus infection, however, inhibits the development of suppressor T-cell dependent

tolerance in mice, with respect to specific IgE-production [17].

Thus, sensitization might also be enhanced by modulation of the immunological response to inhaled allergens due to a preceding viral respiratory tract infection.

However, systemic sensitization was followed by bronchial reaction on specific provocation which was indistinguishable between infected and non-infected animals. Furthermore, the specific bronchial reactions to OA challenge following systemic and inhalative sensitization were comparable when preceded by PI-3 infection. This finding indicates, that PI-3 infection rather influenced mucosal permeability than immune response to the antigen in our model.

In conclusion, PI-3-virus infection of the airways have been shown to enhance inhalative allergic sensitization in the guinea-pig. Mucous membrane damage with increased airway permeability to macromolecules seem to play an important role in the viral induced sensitizing process. The possibility of modulation of the immunological reaction to allergens due to viral infections deserves further investigation.

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