

Virus-induced airway hyperresponsiveness in the guinea pig can be transferred by bronchoalveolar cells

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For the investigation of whether inflammatory cells were responsible for virus-induced airway hyperresponsiveness, tracheal spirals from healthy guinea pigs were incubated in organ baths with different numbers of bronchoalveolar cells obtained from guinea pigs 4 days after their inoculation with parainfluenza-3 (P-3) virus or control solution. Airway responsiveness was measured by performance of histamine concentration/response (C/R) curves on the tissues. Preparations incubated with 5×10^5 cells/ml obtained from guinea pigs treated with P-3 virus demonstrated a significant upward shift of the histamine C/R curve. The maximal contraction was increased by 26% as compared with the tissues incubated with the same number of cells from animals inoculated with control solution. When the number of cells was increased further to 5×10^6 cells/ml, no additional upward shift of the C/R curve was seen; the increase in maximal contraction was 24%. Tracheal spirals incubated with 5×10^4 cells/ml did not affect the histamine C/R curves. Addition of P-3 virus to the organ bath during the incubation period with the cells did not affect the histamine C/R curve either, irrespective of the inoculation solution or the number of bronchoalveolar cells used. The relative number of alveolar macrophages in bronchoalveolar lavage fluid decreased significantly from $86.3\% \pm 2.6\%$ in the control group to $71.8\% \pm 3.3\%$ in the P-3 virus group as a consequence of a significant increase in the percentage of monocytes, lymphocytes, and eosinophils. These results suggest that bronchoalveolar cells are causally involved in the virus-induced airway hyperresponsiveness. (J ALLERGY CLIN IMMUNOL 1992;90:364-72.)

Key words: Parainfluenza-3 virus, in vitro airway responsiveness, bronchoalveolar cells, histamine

Acute viral infection of the respiratory tract has frequently been described to exacerbate symptoms in patients with asthma and to increase the airway response to inhaled bronchoconstrictor agents.¹⁻³ In healthy persons respiratory virus infections also increase the degree of bronchospastic responses to cholinergic and histaminergic receptor stimulation⁴⁻⁷ and exposure to citric acid and nitrates or cold air during exercise.^{4,7,8} This hyperresponsiveness of the airways develops within 2 days after the initial infection and

Abbreviations used

P-3:	Parainfluenza-3
TCID ₅₀ :	Tissue culture infective dose ₅₀
C/R:	Concentration/response
EC ₅₀ :	Effective concentration provoking half maximal response

lasts for several weeks.^{6,7,9} A number of experiments have been performed in animals to unravel the pathogenesis of the virus-induced airway hyperresponsiveness¹⁰⁻¹²; however, the interactive role of bronchoalveolar cells has never been emphasized.¹³

Recently we developed an animal model in which guinea pigs were inoculated intratracheally with parainfluenza type 3 (P-3) virus: a modification of the model described by Buckner et al.¹⁰ A sustained non-specific airway hyperresponsiveness was found up to 16 days after inoculation and was accompanied by an

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increase in total number of bronchoalveolar cells, predominantly alveolar macrophages.¹⁴ These cells are primarily responsible for the elimination of the virus. In addition, replication of P-3 virus in guinea pig and calf alveolar macrophages *in vitro* and *in vivo* has been reported.¹⁵⁻¹⁷ Furthermore, many giant, foamy macrophages were found in cytopsin preparations of bronchoalveolar lavage fluid and in histologic specimens of the airways of animals treated with P-3 virus, which is an indication for activation of these cells. Indeed, chemiluminescence measurements revealed that P-3 virus potently stimulates, equal to or better than zymosan, bronchoalveolar cells.¹⁸ During stimulation/activation of inflammatory cells an array of mediators can be released that might affect airway responsiveness.

To investigate the role of inflammatory cells in the induction of airway hyperresponsiveness in more detail, various numbers of bronchoalveolar cells obtained from animals inoculated with control solution or P-3 virus were incubated together with tracheas of nontreated healthy animals. Therefore histamine concentration/response (C/R) curves were constructed with or without additional stimulation of the bronchoalveolar cells with P-3 virus in the organ bath. Bronchoalveolar cells were harvested 4 days after inoculation, because earlier studies revealed that changes in airway reactivity and changes in the number of bronchoalveolar cells in the lungs were most pronounced 4 days after P-3 virus inoculation.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Dunkin Hartley (Harlan Olac Ltd., Bicester, Oxon, U.K.) guinea pigs (400 to 500 gm) were housed under controlled conditions in an isolator. Water and commercial food were allowed *ad libitum*. The guinea pigs were without infections of the respiratory airways as evaluated by (a) the health-monitoring quality control report by Harlan Porcellus (United Kingdom) and (b) by histological examination.

Intratracheal inoculation

Bovine P-3 virus was kindly provided by Duphar B.V. (Weesp, The Netherlands). P-3 virus was grown on Madin Darby (ATCC, Rockville, Md.) bovine kidney cells and harvested at 3 to 5 days, depending on the cytopathic effects after the initial inoculation. The titre of virus was $10^{8.9}$ 50% tissue culture infective dose (TCID₅₀) per milliliter.

The inoculation volume was reduced to 0.1 ml, and the virus was suspended in sterile pyrogen-free saline solution to minimize the mechanical effects of the inoculation procedure. The virus suspension was centrifuged at 100,000 g and resuspended in saline solution (in 10 times less volume). Growth medium was handled in the same way. The animals were anaesthetized with ether and were laid on their backs

on a small table. The mandibles were kept open by two elastic rubber rings, and a needle with a rounded head was placed just behind the glottis. The rubber rings were removed, and the animal was placed in an upright position. Subsequently, 0.1 ml of the inoculum was gently injected into the trachea. Such a procedure results in infection with P-3 virus in all animals subjected to the procedure. Control guinea pigs were treated in the same way receiving 0.1 ml sterile control solution. Guinea pigs inoculated with control solution and P-3 virus were housed separately in isolators.

Airway responsiveness *in vitro*

Healthy untreated guinea pigs were killed with an overdose of pentobarbital sodium: 30 mg/100 gm body weight, intraperitoneally (Nembutal containing 60 mg/ml pentobarbital sodium, Abbott Laboratories, North Chicago, Ill.). The tracheas were isolated and dissected free of connective tissue and blood vessels. The tracheas were cut in a spiral and divided into two parts of seven rings each. After the incubation period (see "Experimental protocol" section) one end of a trachea was fixed in the organ bath, the other end was attached to an isotonic transducer (Harvard Apparatus Inc., Natick, Mass.). The tissues were mounted in a siliconized organ bath (8 ml) filled with Krebs bicarbonate buffer (37° C, pH = 7.4) of the following composition (in mmol/L): NaCl, 118.1; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 25.0; KH₂PO₄, 1.2; and glucose, 8.3, which was continuously gassed with a mixture of 5% CO₂ and 95% O₂. Tracheal spirals were kept under a load of 0.8 gm, since previous studies have proved that this was the optimal counterweight to measure contractions and relaxations under these conditions. Contractions of the tissues were displayed on a two-channel pen recorder (Servogor, type 220; BBC Brown Boveri & Cie., Baden, Switzerland).

Lung lavages

Four days after inoculation, the animals received a lethal dose of 30 mg/100 gm body weight pentobarbitone sodium, intraperitoneally. The trachea was trimmed free of connective tissue, and a small incision was made to insert a cannula. Via this cannula the lungs were filled with NaCl-ethylenediamine tetraacetic acid (EDTA) buffer (0.15 mol/L NaCl, 2.6 mmol/L EDTA) *in situ* under a pressure of 30 cm H₂O. The fluid was withdrawn from the lungs after gentle lung massage and collected in a plastic tube on ice. This procedure was repeated until 80 ml of fluid was obtained. Only plastics and siliconized glassware were used throughout the isolation procedure to minimize adherence of the cells to the walls of the tubes. The cells were sedimented at 400 g for 10 minutes at 4° C and rinsed in Krebs bicarbonate. Per group cell suspensions from several animals were pooled and washed twice with Krebs bicarbonate buffer. The cells were stained with a Türk solution and counted in a Bürker-Türk (van der Kup, Utrecht, The Netherlands) bright-line counting chamber. Concentrations of 5×10^{-4} , 5×10^{-5} , or 5×10^{-6} cells/ml were made. Viability was greater than 95% as assessed by trypan blue exclusion.

Cell suspensions were analyzed morphologically after centrifugation on microscope slides. Air-dried preparations

were fixed and stained with Diff-Quik (Merz + Dade A.G., Dürdingen, Switzerland). The cells were differentiated under the light microscope into alveolar macrophages, monocytes, lymphocytes, eosinophils, and neutrophils and expressed as percentage of the total number of cells in the bronchoalveolar lavage fluid.

Experimental protocol

One milliliter of a solution containing different numbers of bronchoalveolar cells (4°C) was added to prewarmed (37°C) organ baths (8 ml) that were continuously gassed with a mixture of 5% CO_2 and 95% O_2 . After a time interval of 10 minutes the tracheas were added to the solution containing the cells for an additional period of 10 minutes; thereafter 200 μl Krebs or P-3 virus was added to the organ bath to stimulate the bronchoalveolar cells. The virus suspension used was first centrifuged at 100,000 g and resuspended in Krebs buffer in two times less volume. This concentration of virus proved to be an appropriate stimulus (equal to, or better than zymosan) for bronchoalveolar cells (1×10^6) as measured by chemiluminescence *in vitro*. After an incubation period of 45 minutes the tracheas were connected to the transducers, and an additional 7 ml prewarmed and pregassed Krebs bicarbonate was added to the organ bath. After an additional stabilization period of 45 minutes (still in the presence of the bronchoalveolar cells) a cumulative C/R curve to histamine (histamine phosphate; Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands) was performed. The proximal or distal part of the tracheal spiral was used alternately for incubation with the various number of cells obtained from both inoculation groups. Only one single number of cells was tested on one tissue segment and only one histamine C/R curve was constructed. The experimental groups were equally distributed over the experimental days.

For the investigation of whether the bronchoalveolar cells from guinea pigs treated with control solution or the virus itself affected the histamine C/R curve, two additional control groups were included consisting of tracheal spirals incubated in 1 ml of Krebs bicarbonate without cells and stimulated with 200 μl Krebs-bicarbonate or P-3 virus suspension; a similar experimental time schedule was used as described above.

After construction of histamine C/R curves, all preparations were maximally relaxed with 0.02 mol/L aminophylline (Theophylline ethylenediamine, Onderlinge Pharmaceutische Groothandel) for the investigation of influences of basal tone on changes in airway contraction. In addition, tracheas were mounted in organ baths according to the standard procedure (0.8 gm counterweight, washed three times, 45-minute stabilization period).¹⁹ After a stable tone was reached, the tracheas were not stimulated, or stimulated with 5×10^5 or 5×10^6 bronchoalveolar cells from control or virus-infected animals, and changes in tone were monitored for 45 minutes.

Histologic examination

In additional experiments, two tracheas per experimental group were fixed for histologic examination after an incu-

bation period of 55 minutes, that is, before the stabilization period of 45 minutes, to evaluate attachment of bronchoalveolar cells to the trachea. The tissues were fixed by immersion in a mixture of 2% paraformaldehyde + 2.5% glutaraldehyde in phosphate buffer for at least 24 hours. After an overnight rinse in the same buffer, supplemented with 7.5% sucrose, the samples were postfixed in 2% OsO_4 (0.05 mol/L veronal acetate buffer, pH 7.4) and processed in the routine way before being embedded in Epon.²⁰ Two-micrometer-thick sections were prepared and stained with toluidine blue. From complete tracheal rings, the serosal and mucosal side were investigated under a microscope for adherence of inflammatory cells.

Statistical analysis

Differences between groups after cumulative C/R curves with histamine on isolated tissues of various groups were tested by two-way analysis of variance (ANOVA). In addition, the C/R curves for histamine were analyzed by means of a computerized curve-fitting technique based on the 4-parameter logistic equation.²¹ The parameters defining the sigmoidal curve, that is the maximal response (in millimeters of contraction), the EC_{50} value (the concentration provoking a half maximal response), and the slope factor were determined for each individual C/R curve. The respective parameters were statistically evaluated with Student's unpaired t test. Effects of (a) pretreatment of the guinea pigs, (b) cell density, and (c) the addition of virus to the organ bath was tested with a multifactorial ANOVA. Differences in the absolute and relative number of bronchoalveolar cells between the guinea pigs inoculated with control solution or P-3 virus were tested with Student's unpaired t test. All p values < 0.05 were considered to reflect a statistically significant difference.

RESULTS

Airway responsiveness *in vitro*

In the absence of bronchoalveolar cells, tracheas obtained from healthy guinea pigs showed a concentration-dependent increase in contraction to incremental concentrations of histamine (Fig. 1). Similar histamine C/R curves were constructed on tracheas incubated in the presence of different numbers of bronchoalveolar cells obtained from animals inoculated with control solution (Fig. 2). However, when incubated in the presence of 5×10^5 cells harvested from guinea pigs inoculated with P-3 virus, the histamine C/R curve was significantly shifted upward as compared with the curve constructed in the presence of a similar number of cells obtained from animals inoculated with control solution ($p < 0.01$, two-way ANOVA, Fig. 2, B). The maximal contraction was increased by 26% ($p < 0.01$, Table I). A cell density of 5×10^6 bronchoalveolar cells did not induce an additional upward shift of the C/R curve (Fig. 2, C). The EC_{50} value was not significantly decreased (Table I). Incubation of tracheas with 5×10^4 cells obtained

from guinea pigs inoculated with P-3 virus induced no change in the histamine C/R curve as compared with that obtained after inoculation with cells from animals inoculated with control solution (Fig. 2, A). A significant effect was observed of pretreatment of the animals and the cell density in the organ bath ($p < 0.000$, $f = 38.610$, $df = 1$ and $p < 0.002$, $f = 5.017$, $df = 3$, respectively, multifactorial ANOVA). Addition of P-3 virus to the organ bath during the incubation period with bronchoalveolar cells did not affect the histamine C/R curve in excess ($p < 0.612$, $f = 0.268$, $df = 1$, multifactorial ANOVA). In the absence or presence of P-3 virus, a similar contractile response was seen after incubation of the tracheas with 5×10^4 cells obtained from animals inoculated with control solution or P-3 virus (Fig. 3, A). The histamine C/R curves were again shifted upward significantly after incubation with 5×10^5 or 5×10^6 cells obtained from animals inoculated with virus ($p < 0.01$, two-way ANOVA, Figs. 3, B and C), and the maximal responses were increased by 24% and 36% ($p < 0.01$, Table II), respectively.

Histamine C/R curves constructed on tissues incubated in the presence of P-3 virus without bronchoalveolar cells (Fig. 1), did not differ from those merely in the absence of P-3 virus (Fig. 1), or with cells obtained from guinea pigs inoculated with control solution (Figs. 2 and 3). Likewise, the maximal contractions were not different between those experimental groups (Tables I and II).

The various incubation procedures did not affect the basal tone of the tracheas, since no difference in maximal relaxation, as calculated from basal tone, was observed between the experimental groups after aminophylline stimulation.

Furthermore, addition of bronchoalveolar cells from control or virus-infected animals to nontreated tracheas that had already been equilibrated for 45 minutes did not result in a significant change in basal tone in the following 45 minutes.

Total and differential cell counts

The total number of bronchoalveolar cells isolated from the airways of guinea pigs 4 days after inoculation with the control solution was $9.19 \pm 1.23 \times 10^6$ cells/lung ($n = 7$). This number was significantly increased to $20.60 \pm 1.63 \times 10^6$ cells/lung ($n = 7$, $p < 0.01$) in animals inoculated with P-3 virus; the increase was predominantly due to the larger absolute number of alveolar macrophages (7.90 ± 0.91 control solution and $14.70 \pm 1.01 \times 10^6$ P-3 virus; $p < 0.01$). Despite the increase in absolute number, the relative number of al-

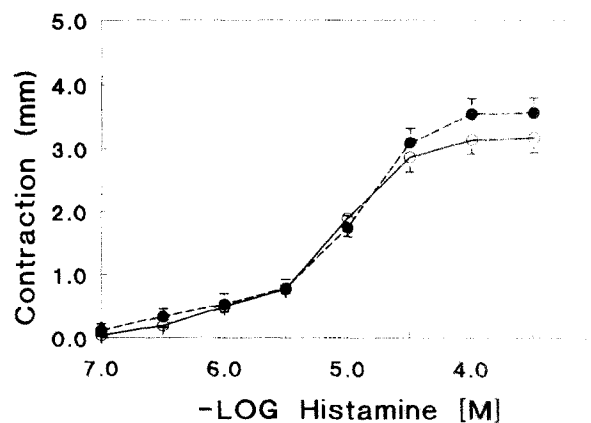


FIG. 1. C/R curve for histamine on isolated tracheal spirals from healthy guinea pigs after incubation with 200 μ l Krebs solution (open circles) or P-3 virus (closed circles) without bronchoalveolar cells. Results are presented as mean $\pm$ SEM. The various parameters derived from these curves as well as the number of experiments are shown in Table II. C/R curves for histamine were similar in both groups (two-way ANOVA).

veolar macrophages in the animals inoculated with P-3 virus was decreased as compared with that of the ones inoculated with control solutions (control solution and P-3 virus inoculation: $86.27\% \pm 2.64\%$ and $71.83\% \pm 3.25\%$, $p < 0.01$). An increase in the relative number of lymphocytes ($2.42\% \pm 0.54\%$ and $5.14\% \pm 0.82\%$, respectively, $p < 0.05$); monocytes ($1.02\% \pm 0.22\%$ vs $3.06\% \pm 0.25\%$ respectively, $p < 0.01$) and eosinophils ($6.20\% \pm 1.87\%$ vs $14.8\% \pm 3.3\%$ respectively, $p < 0.05$) was observed after treatment with virus (Fig. 4). The relative number of neutrophils did not change between the control solution and P-3 virus group ($5.01\% \pm 1.95\%$ and $5.13\% \pm 1.14\%$, respectively, Fig. 4).

Histologic examination

Histologic evaluation revealed that a 55-minute incubation period of bronchoalveolar cells with the trachea did not result in adherence of the cells to the tissue. Therefore no quantitative calculations were performed. At the end of the experimental period the viability of the bronchoalveolar cells in the incubation medium was still greater than 95%.

DISCUSSION

This study showed that bronchoalveolar cells obtained from guinea pigs infected with virus, increased in a concentration-dependent manner the responsiveness of isolated tracheal spirals to histamine. Increasing concentrations of bronchoalveolar cells obtained

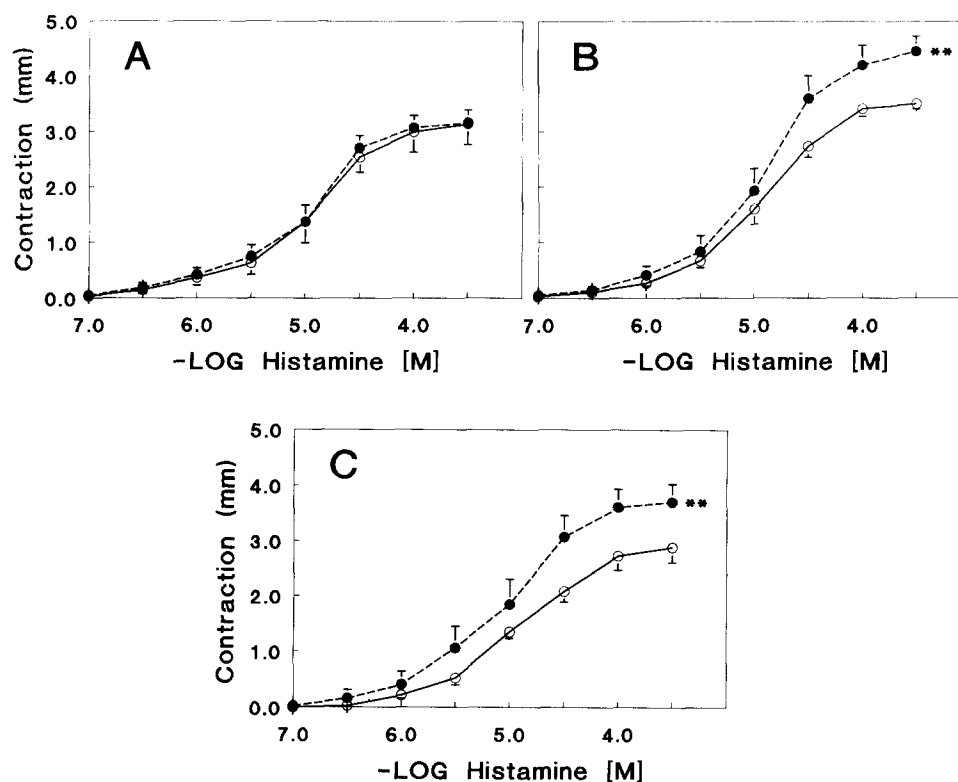


FIG. 2. C/R curve for histamine on isolated tracheal spirals from healthy guinea pigs after incubation with 5×10^4 (A), 5×10^5 (B), or 5×10^6 (C) bronchoalveolar cells/ml obtained from animals 4 days after inoculation with control solution (open circles) or P-3 virus (closed circles). Results are presented as mean $\pm$ SEM. The parameters derived from these curves as well as the number of experiments are shown in Table I. Histamine C/R curves of tracheal spirals were shifted significantly upward after incubation with 5×10^5 or 5×10^6 bronchoalveolar cells/ml obtained from guinea pigs inoculated with P-3 virus as compared with cells obtained from animals inoculated with control solution (** $p < 0.01$, two-way ANOVA).

TABLE I. Various parameters* derived from histamine C/R curves on isolated tracheal spirals after incubation with bronchoalveolar cells from guinea pigs inoculated with P-3 virus

Treatment	Maximal contraction (mm)	EC ₅₀ ($\times 10^{-5}$ mol/L)	Slope factor	No.
5×10^4 Cells				
Control solution	3.22 ± 0.42	1.20 ± 0.17	1.50 ± 0.25	6
P-3 virus	3.28 ± 0.25	1.10 ± 0.25	1.40 ± 0.22	6
5×10^5 Cells				
Control solution	3.64 ± 0.11	1.30 ± 0.24	1.40 ± 0.27	5
P-3 virus†	$4.60 \pm 0.32‡$	1.30 ± 0.28	1.28 ± 0.17	5
5×10^6 Cells				
Control solution	3.08 ± 0.38	1.40 ± 0.24	1.16 ± 0.16	5
P-3 virus†	3.81 ± 0.37	1.10 ± 0.24	1.28 ± 0.15	5

*Parameters were calculated by means of a computerized analysis of individual curves based on the 4-parameter logistic equation. Results are expressed as means $\pm$ SEM.

† $p < 0.01$; Curves were significantly shifted upward as calculated by a two-way ANOVA.

‡ $p < 0.01$; Significantly different from the respective control solution group (Student's unpaired t test).

from animals inoculated with control solution did not affect the histamine C/R curve; the C/R curves were similar to those obtained from tissues incubated in the absence of cells. The total number of bronchoalveolar

cells isolated from the airways of animals inoculated with virus was increased as compared with the number of cells harvested from guinea pigs inoculated with control solution; the relative numbers of the different

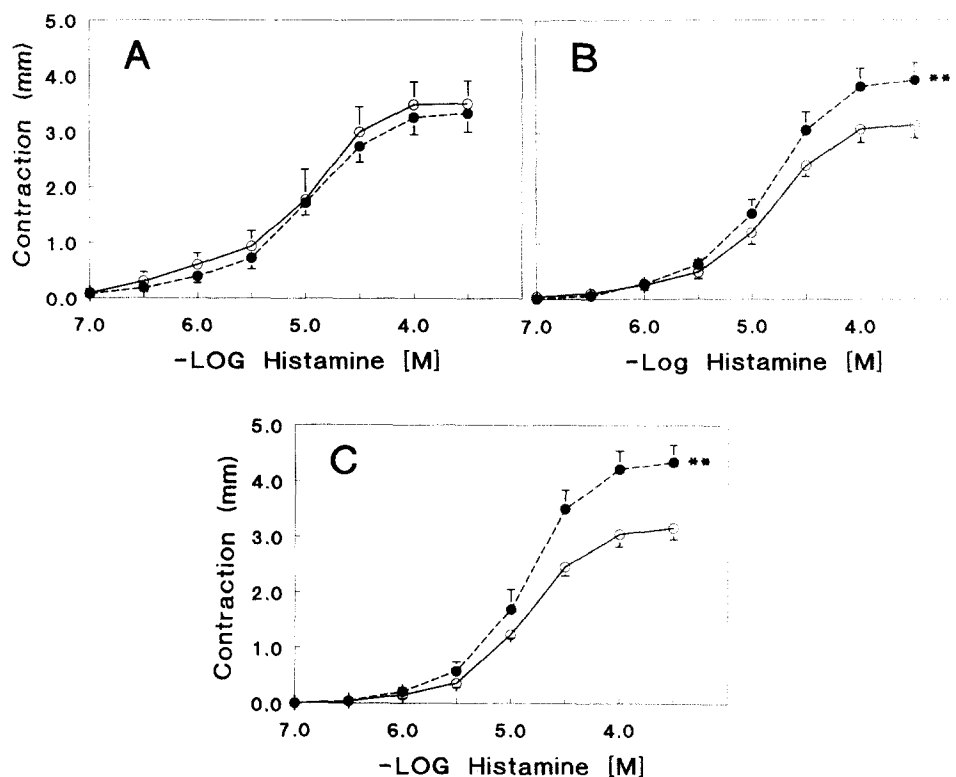


FIG. 3. C/R curve for histamine on isolated tracheal spirals from healthy guinea pigs after incubation with P-3 virus and 5×10^4 (A), 5×10^5 (B), or 5×10^6 (C) bronchoalveolar cells/ml obtained from animals 4 days after inoculation with control solution (open circles) or P-3 virus (closed circles). Results are presented as the mean $\pm$ SEM. The parameters derived from these curves as well as the number of experiments are shown in Table II. A similar upward shift of the histamine C/R curves was seen as in Fig. 2 after incubation with bronchoalveolar cells obtained from guinea pigs inoculated with P-3 virus in the presence of 200 μ l of Krebs buffer. (** $p < 0.01$, two-way ANOVA, control solution versus P-3 virus inoculation).

TABLE II. Various parameters* derived from histamine C/R curves on isolated tracheal spirals after incubation with bronchoalveolar cells from guinea pigs inoculated with P-3 virus and additional stimulation with P-3 virus in the organ bath

Treatment	Maximal contraction (mm)	EC ₅₀ ($\times 10$ mol/L)	Slope factor	No.
5×10^4 Cells				
Control	3.62 $\pm$ 0.44	1.10 $\pm$ 0.30	1.11 $\pm$ 0.21	4
P-3 virus	3.46 $\pm$ 0.39	1.40 $\pm$ 0.20	1.22 $\pm$ 0.17	5
5×10^5 Cells				
Control	3.29 $\pm$ 0.25	1.50 $\pm$ 0.24	1.29 $\pm$ 0.17	5
P-3 virus†	4.09 $\pm$ 0.31	1.40 $\pm$ 0.14	1.25 $\pm$ 0.06	5
5×10^6 Cells				
Control	3.23 $\pm$ 0.23	1.60 $\pm$ 0.14	1.44 $\pm$ 0.10	7
P-3 virus†	4.40 $\pm$ 0.33‡	1.40 $\pm$ 0.19	1.66 $\pm$ 0.14	7
Krebs buffer§	3.32 $\pm$ 0.23	0.77 $\pm$ 0.08	1.19 $\pm$ 0.13	5
P-3 virus§	3.68 $\pm$ 0.34	1.10 $\pm$ 0.26	1.12 $\pm$ 0.29	4

*Parameters were calculated by means of computerized analysis of individual curves based on 4-parameter logistic equation. Results expressed as means $\pm$ SEM.

† $p < 0.01$; Curves shifted significantly by upward as calculated by two-way ANOVA.

‡ $p < 0.01$; Significantly different from the respective control solution group (Student's unpaired t test).

§Tracheal spirals incubated in presence of Krebs buffer or P-3 virus in organ bath, but in the absence of bronchoalveolar cells.

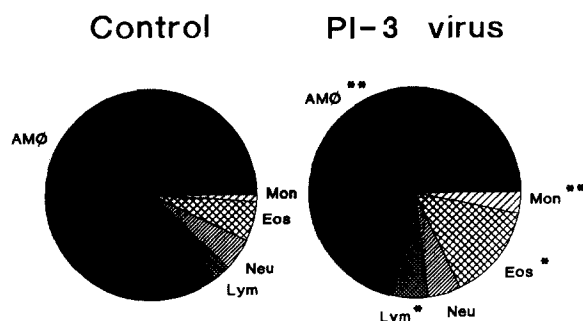


FIG. 4. Mean distribution of bronchoalveolar cells obtained by lung lavage from guinea pigs 4 days after inoculation with control solution or P-3 virus. Relative number of alveolar macrophages (*AMØ*) was significantly decreased in the group treated with P-3 virus, whereas the relative number of eosinophils (*Eos*), lymphocytes (*Lym*), and monocytes (*Mon*) was increased. No difference in the relative number of neutrophils (*Neu*) was seen between the two groups. Student's unpaired *t* test; **p* < 0.05, ***p* < 0.01.

types of bronchoalveolar cells were changed as well.

It has been accepted widely that inflammatory cells may contribute to the induction of airway hyperresponsiveness.²²⁻²⁴ However, the role of inflammatory cells in the virus-induced airway hyperresponsiveness has been poorly investigated, although it is known that several inflammatory cell types are involved in the elimination of the virus. We showed previously that intratracheal inoculation of P-3 virus in to guinea pigs resulted 4 days (but not 2 days) later in an increased responsiveness of the trachea to histamine.²⁵ It is interesting to note that an increased number of inflammatory cells was found 4 days after inoculation with P-3 virus but not after 2 days. Therefore an association between the number of inflammatory cells and the induction of airway hyperresponsiveness was suggested. Moreover, giant, foamy macrophages were found in cytospin preparations of bronchoalveolar lavage fluid and in histologic specimens of the lungs in animals treated with P-3 virus, indicating activation of these cells *in vivo*.

Both the activation and the number of inflammatory cells in the lungs, and the subsequent mediator release, may be important in the induction of airway hyperresponsiveness. In the present study the number of cells was more than doubled in animals treated with virus. This was due primarily to an increase in the number of alveolar macrophages; in addition, the number of monocytes, lymphocytes, and eosinophils had increased. The quantity of basal mediator release by bronchoalveolar cells in airways may be assumed to rise with the number of cells. Therefore it can be argued that the increase in basal mediator release

caused by the increase in the number of inflammatory cells, triggers the induction of an increased airway responsiveness. However, no evidence for such a hypothesis is found in the present study, since incubation of tracheas with increasing numbers of bronchoalveolar cells, obtained from animals inoculated with control solution, did not change the responsiveness to histamine.

Comparative results were obtained by Tamaoki et al.²⁶ They added 2×10^7 alveolar macrophages to canine bronchial rings but did not find any changed reactivity in response to exogenously applied acetylcholine. Furthermore, it was found that addition of increasing numbers of granulocytes up to 5×10^6 cells to human airway segments did not influence the sensitivity to methacholine; in contrast, there was even a decreased sensitivity with higher numbers of cells.²⁷ This observation corroborates our previous results¹⁹; although an increase in the number of bronchoalveolar cells was detected in the guinea pig lung 24 hours after an endotoxin aerosol, a *hypo*responsiveness to histamine was found *in vivo*. Thus the *number* of bronchoalveolar cells seemed not directly responsible for the induction of airway hyperresponsiveness.

It has been demonstrated that P-3 virus replicates in alveolar macrophages *in vitro* and *in vivo*.¹⁵⁻¹⁷ Therefore the increased airway responsiveness observed after incubation with bronchoalveolar cells obtained from animals treated with virus may be induced by P-3 virus released from these cells. However, incubation of the tissues in the presence of P-3 virus with or without bronchoalveolar cells did not affect tracheal responsiveness and, so, rules out this possibility. The latter findings are rather remarkable, since we found that 1×10^6 bronchoalveolar cells could be stimulated by the same amount of virus, thereby enhancing the production of reactive oxygen radicals *in vitro*. However, such a stimulation and/or the kind of mediators released by the cells obtained from animals inoculated with control solution may not be appropriate to affect airway responsiveness. Furthermore, it was demonstrated that the superoxide production by bronchoalveolar cells, isolated from animals 4 days after P-3 virus inoculation, was significantly decreased by 36% on additional stimulation with P-3 virus. Thus cells that have already been stimulated with P-3 virus *in vivo* responded less on additional stimulation with P-3 virus *in vitro*.²⁸ This might account for the absence of an additive increase in airway contraction as found in the tissues incubated with cells obtained from animals treated with P-3 virus and additionally stimulated with P-3 virus.

Finally, a possible explanation for the induction of airway hyperresponsiveness may be found in the re-

lease of other additional mediator(s) by cells infected with virus. In a number of reviews it has been emphasized that inflammatory cells can release an array of mediators that may directly or indirectly contribute to airway hyperresponsiveness.^{22-24,29} These mediators are approximately divided into four groups. First, reactive oxygen radicals are generated by various inflammatory cells. These products induce airway contraction *in vitro* and *in vivo* and induce airway hyperresponsiveness in cats *in vivo*.³⁰⁻³¹ However, in the present study histologic examination revealed that the bronchoalveolar cells did not adhere to the tracheas. Therefore oxygen species are probably not involved because they are not released in close vicinity of the tracheas. In addition, oxygen radicals are rapidly inactivated by their reaction with other molecules, such as oxygen, in the organ bath that are abundantly present. Second, inflammatory cells release a number of proteases that may alter structural elements within the tissues. This can lead to a change in basal tone and subsequently to a change in airway responsiveness. However, in this study it is demonstrated that the basal tone is not influenced by bronchoalveolar cells. Therefore it is not likely that proteases are involved in the induction of airway hyperresponsiveness. Third, cytokines such as interleukins, interferons, and tumor necrosis factor, may be involved because they are found to be synthesized after viral infections and are implicated in the induction of airway hyperresponsiveness.³² Finally, a number of inflammatory cell types can produce phospholipid mediators, such as platelet activating factor, cyclooxygenase products, and lipoxygenase products after stimulation.²¹⁻²³ Infection with virus of alveolar macrophages *in vivo* and *in vitro*, the major cell found in bronchoalveolar lavage fluid, results in profound metabolic and functional changes in these cells.³³⁻³⁴ Leagreid et al.¹⁵ showed that P-3 virus application to cultured alveolar macrophages resulted 4 days later in an increased release of arachidonic acid metabolites. This was associated with the maximal expression of viral antigen and the release of infectious P-3 virus by the cultured alveolar macrophages. This temporal association suggests that viral replication might be required to promote arachidonate metabolism and to release other mediators by inflammatory cells. Changes in the phospholipase A₂ activity by viruses seemed not to be restricted to macrophages, since an enhanced phospholipase A₂ activity has been demonstrated in lymphocytes from humans with an influenza virus infection.³⁵ In this context, it was recently shown that incubation of human tracheal segments with (sub)threshold concentrations of inflammatory mediators, such as histamine, the stable thromboxane A₂

analogue U46619, prostaglandin D₂, or F_{2α} all increase the responsiveness to methacholine.³⁶ It cannot be excluded that threshold doses of mediators released by inflammatory cells trigger second messenger systems within the cell, leading to an increased response after additional stimulation with histamine and probably other drugs, resulting in an increased maximal contraction of the respiratory airways.

In conclusion, changes in mediator release rather than in the number of inflammatory cells may be responsible for the virus-induced airway hyperresponsiveness. Whether one, more, or a mutual interaction between cells and mediators is (are) involved remains to be determined.

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