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Virus-induced airway hyperresponsiveness in the guinea-pig: possible involvement of histamine and inflammatory cells

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1 Guinea-pig tracheal contractions by histamine and by the cholinoceptor agonist, arecoline, are significantly enhanced (30% and 20%, respectively), 96 h after intra-tracheal inoculation with Parainfluenza-3 (PI-3) virus.

2 The airway hyperresponsiveness in animals inoculated with virus coincides with a significant increase in the number of broncho-alveolar cells (82%), and in the albumin concentration (121%) in lung lavage fluid, relative to values obtained in guinea-pigs challenged with control solution.

3 The chemiluminescence production by isolated broncho-alveolar cells, obtained from virus-infected guinea-pigs 96 h after inoculation stimulated with PI-3 virus *in vitro*, is significantly reduced by 42% relative to broncho-alveolar cells obtained from animals inoculated with control solution. This diminution was not specific for stimulation by PI-3 virus since the chemiluminescence production was also significantly reduced by 30% in response to zymosan.

4 Pretreatment of the guinea-pigs with the anti-allergic drugs, oxatomide (2.5 mg kg^{-1}) or nedocromil (2.5 mg kg^{-1}), or the specific H_1-histamine receptor antagonist, levocabastine (0.25 mg kg^{-1}), administered intra-peritoneally twice a day for five successive days, inhibits the virus-induced airway hyperresponsiveness, suppresses the influx of broncho-alveolar cells and increase in albumin content, and corrects the reduced chemiluminescence production by broncho-alveolar cells in response to zymosan.

5 In contrast, the cyclo-oxygenase inhibitor, suprofen (5.0 mg kg^{-1}), the 5-HT_2 receptor antagonist, ketanserin (0.63 mg kg^{-1}), or the Ca^{2+} overload blocker, flunarizine (2.5 mg kg^{-1}) do not modify the above mentioned processes.

6 The platelet-activating factor receptor antagonist, WEB 2170 (10 mg kg^{-1}), reduces virus-induced airway hyperresponsiveness and influx of broncho-alveolar cells into the lungs but does not attenuate the increase of albumin in the bronchial lavage fluid.

7 Guinea-pigs nebulized with histamine, twice a day (30 min) during 4 successive days, do not demonstrate an increased airway responsiveness, but instead show tachyphylaxis in response to histamine *in vitro*. In addition, no influx of inflammatory cells is found in these animals.

8 These results suggest that histamine does not directly increase the responsiveness of the guinea-pig trachea; however, histamine may be involved in a cascade of events leading to airway hyperresponsiveness after a viral infection, a process that could be related to an influx and/or an activation of broncho-alveolar cells after PI-3 virus stimulation.

Keywords: Guinea-pig airways; Parainfluenza 3 virus; viral respiratory infections; tracheal responsiveness; inflammatory cell counts; albumin; chemiluminescence; oxatomide

Introduction

Children infected with parainfluenza or respiratory syncytial virus are prone to develop bronchiolitis, airway hyperresponsiveness, asthma, or abnormalities of pulmonary function (Minor *et al.*, 1974; Glezen *et al.*, 1982; Chanock & McIntosh, 1985; Busse, 1990). Approximately 40% of acute asthmatic attacks in children are associated with acute viral respiratory infections (McIntosh *et al.*, 1973). Moreover, acute viral respiratory infections may initiate development of bronchial hyperresponsiveness in normal as well as in asthmatic patients (Empey *et al.*, 1976; Laitinen & Kava, 1980). Such an airway hyperresponsiveness often develops within two to three days after manifestation of symptoms expressing viral infections, and can persist several weeks thereafter (Little *et al.*, 1978; Aquilina *et al.*, 1980).

Despite efforts, the mechanism underlying this virus-induced airway hyperresponsiveness and the possible role of the number and/or activity of inflammatory cells in the lungs remain to be clarified. Recently, we developed a model in guinea-pigs in which a long-lasting airway hyperresponsiveness was documented *in vitro* and *in vivo* after viral respiratory tract infection (Folkerts *et al.*, 1992a,b). The increased airway contraction of histamine (H_1) or muscarinic receptor stimulation, in such pathological conditions compared to control animals, was associated with an increased number of cells in the broncho-alveolar lavage fluid up to 16 days after intra-tracheal inoculation of Parainfluenza-3 (PI-3) virus, effects being most pronounced 4 days after infection (Folkerts *et al.*, 1992a,b). Furthermore, we found that PI-3 virus is a potent stimulus for broncho-alveolar lavage cells *in vitro*. Indeed, broncho-alveolar cells obtained from virus-infected animals, display a significantly reduced capacity to generate reactive oxygen metabolites upon additional PI-3 virus stimulation *in vitro*, reflecting stimulation and exhaustion *in vivo* (Folkerts *et al.*, 1992a,b).

The purpose of the present study was to elucidate further the importance of inflammatory cell number, type, and function as well as that of various allergic mediators in the airway hyperresponsiveness following viral infections. For this purpose virus-infected guinea-pigs were pretreated with a number of drugs that interfere with the synthesis and/or effect of various autocoids. To quantify such pharmacological modulation, we measured (a) tracheal responsiveness to histamine and the cholinoceptor agonist, arecoline

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in vitro, (b) the number and types of broncho-alveolar cells, (c) albumin concentration in lung lavage fluid, and (d) broncho-alveolar cells function/activity *in vitro* by means of chemiluminescence production after stimulation with PI-3 virus, or with zymosan.

Methods

Animals

Specified-pathogen-free guinea-pigs (400–500 g, male Dunkin Hartley, Harlan Olac Ltd, England) were housed in isolators under controlled conditions. Water and commercial chow were allowed *ad libitum*. The guinea-pigs were free of respiratory airway infections as assessed by the health monitoring quality control report by Harlan Percellus (England), and by histological examination.

Intra-tracheal inoculation

Suspensions of virus (1 ml; Tissue Culture Infective Dose $(TCID)_{50} = 10^{8.9}\ ml^{-1}$) were centrifuged at 100,000 *g* and resuspended in a 1/10 reduced volume (0.1 ml) of sterile pyrogen-free saline in order to minimize possible mechanical effects of the inoculation procedure. Growth medium was subjected to a similar procedure in order to serve as a control solution.

The animals were anaesthetized with ether and were placed in a supine position on a small table. The jaws were kept open by two elastic rubber rings and a needle with a bulbous tip was placed just behind the glottis. The rubber rings were removed and the animal was placed in an upright position. Thereafter, 0.1 ml of the inoculum was gently injected in the trachea. Such a procedure results in infection with PI-3 virus in all animals subjected to the procedure (Folkerts *et al.*, 1992a,b). Control guinea-pigs, housed in a separate isolator, were treated in the same way with 0.1 ml of a control solution.

Bovine Parainfluenza 3 virus (PI-3) was kindly provided by Duphar B.V. (Weesp, The Netherlands).

Experimental protocol

Guinea-pigs were pretreated with pharmacological agents intra-peritoneally (i.p.), twice a day (08 h 00 min and 17 h 30 min) for five successive days, starting on day 0 and ending on day 4. On day 1 the guinea-pigs were inoculated with the control solution or PI-3 virus at 10 h 00 min and received an additional dose of the pharmacological agent at 12 h 00 min. On day 5, the animals received no antagonist and were killed. Drugs were injected into the right or left side of the abdomen alternately.

Pharmacological agents

Drugs were dissolved in β-hydroxypropyl-cyclodextrine solution (20% w/v in diluted water). The sham group received this solvent ($1.0\ ml\ kg^{-1}$) in the same schedule as the drug-treated animals. The drugs used were: suprofen ($5.0\ mg\ kg^{-1}$), a cyclo-oxygenase inhibitor (De Clerck *et al.*, 1975); flunarizine ($2.5\ mg\ kg^{-1}$), a Ca^{2+} overload blocker (Todd & Benfield, 1989); ketanserin ($0.63\ mg\ kg^{-1}$), a 5-HT_2 receptor antagonist (De Clerck *et al.*, 1984); nedocromil ($2.5\ mg\ kg^{-1}$), anti-allergic compound (Auty, 1986); oxatomide ($2.5\ mg\ kg^{-1}$), an anti-allergic compound with H_1 histamine receptor blocking properties (Awouters *et al.*, 1979; De Clerck *et al.*, 1981); WEB 2170 (Bepafant; $10\ mg\ kg^{-1}$), a platelet-activating factor (PAF) receptor antagonist (Heuer *et al.*, 1990); levocabastine ($0.25\ mg\ kg^{-1}$), a specific H_1 histamine receptor antagonist (Van Wauwe, 1989).

Airway responsiveness in vitro

Tracheal responsiveness was measured as described by Folkerts *et al.* (1989). Briefly, guinea-pigs were killed with an overdose of pentobarbitone sodium (Nembutal, 30 mg $100\ g^{-1}$ body weight, i.p.). Trachea were dissected free of connective tissue and blood vessels and were mounted in a siliconized organ bath filled with Krebs-bicarbonate buffer (37°C, pH = 7.4) of the following composition (mM): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 8.3, which was continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. Each trachea was cut in a spiral, and divided into two parts of seven rings each. One end of a trachea was fixed in the organ bath, the other end was attached to an isotonic-transducer (Harvard Bioscience). Tracheal spirals were kept under a load of 0.8 g throughout the experiment. All tissues were washed three times at 15 min intervals, after which a stable tone was reached (within 45 min). The proximal or distal part of the tracheal spiral was used alternatively for the construction of histamine or arecoline cumulative concentration-response (C/R) curves to avoid possible differences between parts. Only one C/R curve was obtained on a tissue segment. Tracheal contractions were displayed on a two-channel pen recorder (Servogor, type 220).

Lung lavages

In order to perform lung lavages, several groups of animals were killed by the administration of $30\ mg\ 100\ g^{-1}$ body weight pentobarbitone sodium (i.p.). Tracheae were trimmed free of connective tissue and a small incision was made for insertion of a cannula into the trachea (Folkerts *et al.*, 1988). The lungs were filled with 12 ml NaCl-EDTA-buffer (0.15 M NaCl, 2.6 mM EDTA) *in situ* under a pressure of 30 cmH_2O. After gentle lung massage for 1 min, 7 ml fluid was withdrawn from the lungs for further processing to determine the albumin content (see albumin content). Such a procedure was repeated without notion of the amount of fluid recovered from the lungs, until 80 ml fluid was obtained from each animal. Cells were then sedimented by centrifugation at 400 *g* for 10 min at 4°C, and washed twice with Krebs-bicarbonate buffer. The cells were stained with Türk's solution and counted in a Bürker-Türk bright-line counting chamber.

All cell preparations were analysed morphologically after centrifugation on microscope slides. Air dried preparations were fixed and stained with Diff-Quik (Merz + Dade A.G., Düdingen, Switzerland). The cells were differentiated into alveolar macrophages, monocytes, lymphocytes, eosinophils and neutrophils by light microscopical observation. Viability was >95% as assessed by the trypan blue exclusion test.

Only plastics and siliconized glassware were used throughout the isolation procedure in order to minimize adherence of the cells to the walls of the tubes.

Albumin content

In the first 7 ml of the lung lavage fluid the content of albumin was measured. For that purpose tubes were centrifuged at 400 *g*, the cell-pellet was resuspended in Krebs and added to the respective cell-pool that was recovered from the 80 ml washing (see lung lavage). The supernatant was stored at −80°C. After thawing, fluids were again centrifuged at 1000 *g*, and in the resultant supernatant the albumin concentration was measured in a Cobas-Bio centrifugal analyzator (Hoffman-LaRoche/Co., Limited Company, Diagnostiga, Basel, Switzerland) with bromocresol green (Boehringer Mannheim GmbH, Mannheim, FRG) as reagent (Doumas *et al.*, 1971).

Table 1 Various parameters† derived from histamine concentration-response curves on isolated tracheal spirals from guinea-pigs pretreated with pharmacological probes and inoculated with control solution or PI-3 virus

Treatment	*Dose* (mg kg^{-1})	*Maximal contractions* (mm)	EC_{50} ($\times 10^{-6}$ M)	*Slope factor*	n
Sham	1.0 ml				
Control		3.5 ± 0.2	4.0 ± 1.0	1.12 ± 0.12	11
PI-3**		4.6 ± 0.3§§	4.4 ± 1.0	1.09 ± 0.06	10
Suprofen	5.0				
Control		3.0 ± 0.3	2.6 ± 0.4	0.87 ± 0.09	6
PI-3**		3.8 ± 0.2§	3.4 ± 0.3	0.93 ± 0.05	6
Oxatomide	2.5				
Control		3.2 ± 0.1	11.0 ± 2.1	1.92 ± 0.25	10
PI-3**		2.7 ± 0.2§	13.0 ± 3.0	2.02 ± 0.34	7
Nedocromil	2.5				
Control		3.7 ± 0.3	2.6 ± 0.9	0.87 ± 0.11	4
PI-3		3.7 ± 0.5	2.4 ± 0.6	1.02 ± 0.06	5
Flunarizine	2.5				
Control		2.1 ± 0.3	8.2 ± 2.2	0.89 ± 0.08	6
PI-3**		3.0 ± 0.1§	7.4 ± 2.9	0.71 ± 0.06	6
Ketanserin	0.63				
Control		3.4 ± 0.4	3.0 ± 0.8	0.89 ± 0.09	4
PI-3**		4.3 ± 0.3	2.5 ± 0.1	1.02 ± 0.03	4
WEB 2170 BS	10.0				
Control		3.2 ± 0.2	2.2 ± 0.3	0.96 ± 0.04	6
PI-3		3.5 ± 0.5	2.1 ± 0.4	0.95 ± 0.08	6
Levocabastine	0.25				
Control		3.0 ± 0.2	4.7 ± 0.7	0.79 ± 0.02	6
PI-3		3.2 ± 0.3	4.7 ± 0.6	0.80 ± 0.03	6

**$P<0.01$; the curves were significantly shifted upwards or downward as calculated by a two-way analysis of variance (ANOVA).
†The parameters were calculated by means of a computerized analysis of the individual curves based on the 4-parameter logistic equation. Results are expressed as means ± s.e.mean.
§$P<0.05$; §§$P<0.01$: significantly different from the respective control group, Student's t test (unpaired).
n = number of observations.

Table 2 Various parameters† derived from arecoline concentration-response curves on isolated tracheal spirals from guinea-pigs pretreated with pharmacological probes and inoculated with control solution or PI-3 virus

Treatment	*Dose* (mg kg^{-1})	*Maximal contractions* (mm)	EC_{50} ($\times 10^{-7}$ M)	*Slope factor*	n
Sham	1.0 ml				
Control		4.9 ± 0.3	7.2 ± 1.3	0.86 ± 0.05	13
PI-3**		5.9 ± 0.4§	5.8 ± 0.9	0.87 ± 0.05	12
Suprofen	5.0				
Control		4.1 ± 0.4	5.4 ± 0.8	0.82 ± 0.04	5
PI-3**		4.9 ± 0.4	6.3 ± 0.9	0.80 ± 0.02	5
Oxatomide	2.5				
Control		5.0 ± 0.3	7.5 ± 1.3	0.96 ± 0.06	11
PI-3		4.2 ± 0.4	8.5 ± 1.0	0.84 ± 0.05	11
Nedocromil	2.5				
Control		4.4 ± 0.6	4.9 ± 1.4	0.82 ± 0.02	5
PI-3		4.2 ± 0.3	4.1 ± 0.7	0.86 ± 0.04	5
Flunarizine	2.5				
Control		4.9 ± 0.4	12.0 ± 5.0	0.80 ± 0.04	6
PI-3		4.5 ± 0.2	8.0 ± 1.6	0.80 ± 0.02	5
Ketanserin	0.63				
Control		4.7 ± 0.6	4.9 ± 0.8	0.77 ± 0.04	4
PI-3**		5.5 ± 0.3	3.7 ± 0.3	0.81 ± 0.02	4
WEB 2170 BS	10.0				
Control		4.5 ± 0.3	4.6 ± 1.1	0.85 ± 0.04	6
PI-3*		5.1 ± 0.5	4.3 ± 0.6	0.83 ± 0.02	6
Levocabastine	0.25				
Control		4.5 ± 0.4	5.7 ± 0.7	0.78 ± 0.02	6
PI-3*		4.7 ± 0.2	3.8 ± 0.5	0.80 ± 0.03	6

*$P<0.05$; **$P<0.01$; the curves were significantly increased as calculated by a two-way analysis of variance (ANOVA).
†The parameters were calculated by means of a computerized analysis of the individual curves based on the 4-parameter logistic equation. Results are expressed as means ± s.e.mean.
§$P<0.05$; significantly different from the control group, Student's t test (unpaired).
n = number of observations.

Generation of chemiluminescence

The concentration of broncho-alveolar cells was adjusted to give 5×10^6 cells ml^{-1} and 200 µl of this suspension was used to measure chemiluminescence generation in a luminometer (type 1251 LKB Wallac, Turku, Finland) at 37°C in the presence of 0.5 mM lucigenin (50 µl). The virus suspension ($TCID_{50} = 10^{8.9}$ ml^{-1}) was centrifuged at 100,000 *g* and resuspended in Krebs-buffer (in 2 times less volume). Growth medium was treated in the same way and served as a control to determine basal chemiluminescence production. The end volume of every tube was adjusted to 0.5 ml with Krebs-buffer. The broncho-alveolar cells were stimulated with: (A) 200 µl control solution, (B) 200 µl PI-3 virus suspension and (C) 2.5 mg zymosan (preopsonized in 100% pooled guinea-pig serum). Chemiluminescence was measured every 60 s over a 40 min period; the peak value was used for data processing.

Histamine nebulization

In additional experiments, conscious guinea-pigs were exposed to a histamine aerosol for 30 min, twice a day, during 4 days. Histamine (2 mg ml^{-1}) was dissolved in sterile saline and nebulized (1 ml min^{-1}) with an electronic ultrasonic nebulizer (Medix, Mecomfa, Lisse, The Netherlands). Control animals were treated in the same way receiving an aerosol of sterile saline. Before the animals were placed in the perspex chamber (7.5 l), saline or histamine was aerosolized during 2 min in order to fill the system completely. In preliminary experiments it was found that 2 mg ml^{-1} histamine was the dose at which the guinea-pigs could stay in the chamber for 30 min, during 4 successive days, without developing severe bronchoconstriction.

On day 5, tracheal responsiveness to histamine and arecoline was measured, the total numbers of broncho-alveolar cells was determined, and the chemiluminescence production in response to zymosan was registrated.

Statistical analysis

Differences between groups after cumulative C/R curves with histamine and arecoline on isolated tissues were tested by two way analysis of variance (ANOVA). The ANOVA calculates the area under the curve of each single C/R curve. Thereafter, the mean of the AUC of the C/R curves within one group was calculated. Accordingly, the AUC of two different groups were compared with one another. In addition, the C/R curves for histamine and arecoline were analysed by means of a computerized curve fitting technique based on the 4-parameter logistic equation (De Lean *et al.*, 1978). The parameters defining the sigmoidal curve, i.e. the maximal response (in mm contraction), the EC_{50} value (the concentration that gives a half maximal response), and the slope factor were determined for each individual C/R curve. The respective parameters were subsequently averaged for the various experimental groups with respect to the maximal response, the EC_{50}, and slope factor of the C/R curves, and were statistically evaluated with Student's *t* test (unpaired). Differences in the total number or differential cell counts and chemiluminescence production were tested by Student's *t* test (unpaired). All *P* values <0.05 were considered to reflect a statistically significant difference (Siegel, 1956).

Chemicals

Oxatomide, flunarizine, levocabastine, suprofen and ketanserin were gifts from Janssen Research Foundation, Beerse, Belgium. Nedocromil was a gift from Fisons, Loughborough, Leicestershire. WEB 2170 BS was a gift from Boehringer Ingelheim KG, Ingelheim am Rhein, Germany. Nembutal, containing 60 mg ml^{-1} pentobarbitone sodium, was purchased from Abbott Laboratories (North Chicago, IL, U.S.A.). Histamine phosphate, was obtained from the Onder-

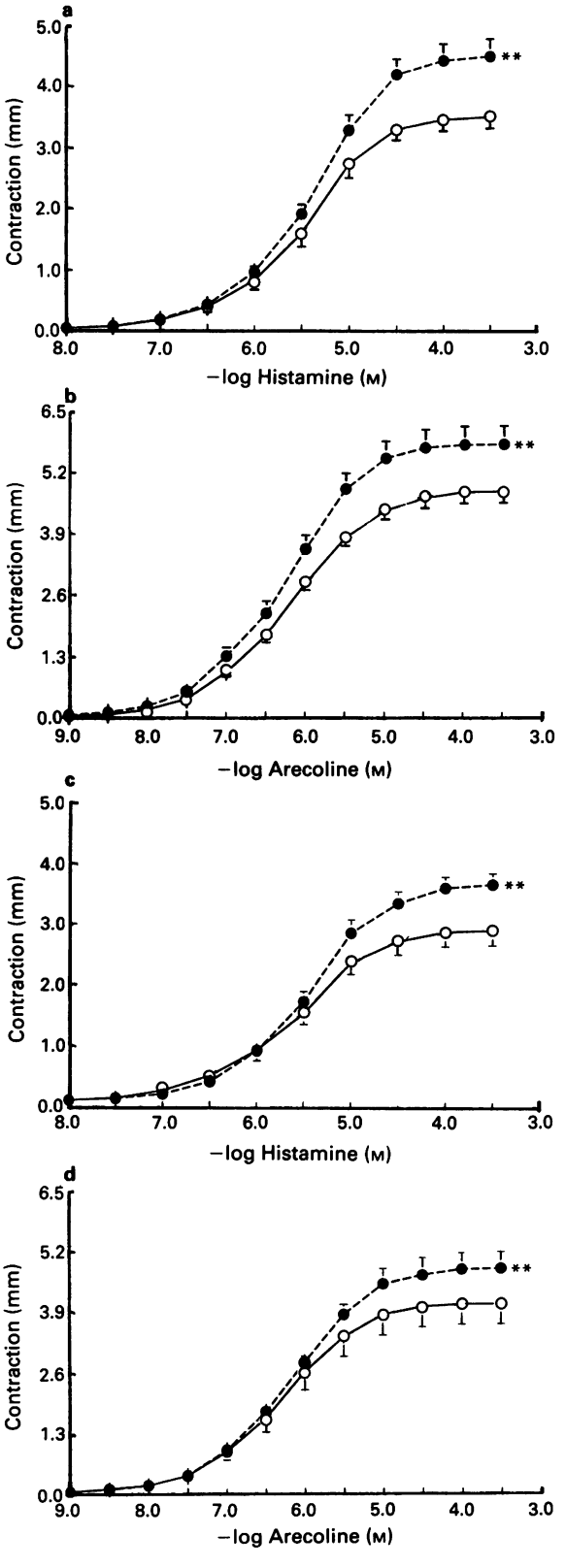

Figure 1 Concentration-response curve for histamine (a and c), and arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreatment with solvent solution (1.0 ml kg^{-1} b.wt., a and b) or suprofen (5.0 mg kg^{-1}, c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. The C/R curves for histamine and arecoline were significantly shifted upwards (**$P<0.01$, two way ANOVA) in the PI-3 virus groups when compared to the control group.

linge Pharmaceutische Groothandel (Utrecht, The Netherlands). Arecoline, lucigenin (*bis*-N-methylacridinium nitrate) and zymosan were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Results

Airway responsiveness in vitro

Concentration/response (C/R) curves for histamine and arecoline on tracheal spirals obtained from animals inoculated with PI-3 virus were significantly ($P<0.01$, ANOVA) shifted upwards as compared to controls (Figure 1a and b, respectively). Maximal contractions induced by histamine were significantly increased by approximately 30% ($P<0.01$, Table 1) and that by arecoline approximately 20% ($P<0.05$, Table 2) in the group inoculated with virus relative to those inoculated with control solution. The EC_{50} value of the arecoline C/R curve was slightly, but not significantly, lower (Table 2) in the virally-infected preparations.

Inhibition of cyclo-oxygenase activity by suprofen, which attenuates the production of all prostanoids derived from arachidonic acid during the time period of the viral-infection, did not affect the airway hyperresponsivess to histamine (Figure 1c, Table 1) and arecoline C/R (Figure 1d, Table 2). The C/R curves for both agonists were shifted upwards ($P<0.01$) in the virus-treated animals.

Treatment of the animals during the viral infection period with the anti-allergic compound oxatomide which has potent H_1-histamine receptor antagonist properties also, or with the mast cell stabilizer nedocromil inhibited the development of tracheal hyperresponsiveness in response to histamine and arecoline (Figure 2, Tables 1 and 2). Treatment with oxatomide reduced the sensitivity to histamine of tracheal preparations, as reflected by a 3 fold shift to the right of the C/R curve relative to that in the sham group ($P<0.01$, Table 1) in both the groups inoculated with control solution and that inoculated with virus. Interestingly, in the PI-3 virus group the histamine C/R curve was additionally shifted downwards ($P<0.05$, ANOVA) as compared with the control group (Figure 2a). The maximal contraction of the virus-inoculated guinea-pigs, after pretreatment with oxatomide, was reduced by more than 40% ($P<0.01$) as compared to the virus-infected sham group (Table 1). Similar to the results obtained with histamine, increased tracheal contractions to arecoline were also inhibited by pretreatment with oxatomide (Table 2; Figure 2b). Treatment with nedocromil inhibited the increase in tracheal contraction to histamine (Figure 2c, Table 1) as well as to arecoline (Figure 2d, Table 2).

Ca^{2+}-overload blockade by flunarazine during the development phase of the virally-induced airway hyperresponsiveness affected the histamine C/R curve in both groups. Maximal responses were decreased ($P<0.01$) and the EC_{50} values were non-significantly increased (Figure 3a, Table 1) as compared to the sham group (Figure 1a, Table 1). Nevertheless, such a pharmacological manipulation did not affect the virus-induced airway hyperresponsiveness. The histamine C/R curve was still shifted upwards in the PI-3 virus group (Figure 3a, $P<0.01$, ANOVA): the maximal contraction being increased by 40% ($P<0.05$, Table 1). However, Ca^{2+}-overload blockade with flunarizine abolished the development of functional hyperresponsiveness to cholinoceptor stimulation, with arecoline as agonist (Figure 3b, Table 2). Blockade of 5-HT_2 binding sites by ketanserin did not affect the virus-induced airway hyperresponsiveness to histamine (Figure 3c, Table 1, $P<0.01$ ANOVA) or arecoline (Figure 3d, Table 2, $P<0.01$ ANOVA) as well.

The PAF-receptor antagonist, WEB 2170, as well as the specific H_1-histamine receptor antagonist, levocabastine, suppressed the increase in maximal tracheal contraction to histamine (Figure 4a and c, Table 1) and to arecoline (Figure 4b and d, Table 2). While the histamine C/R curves on tracheae

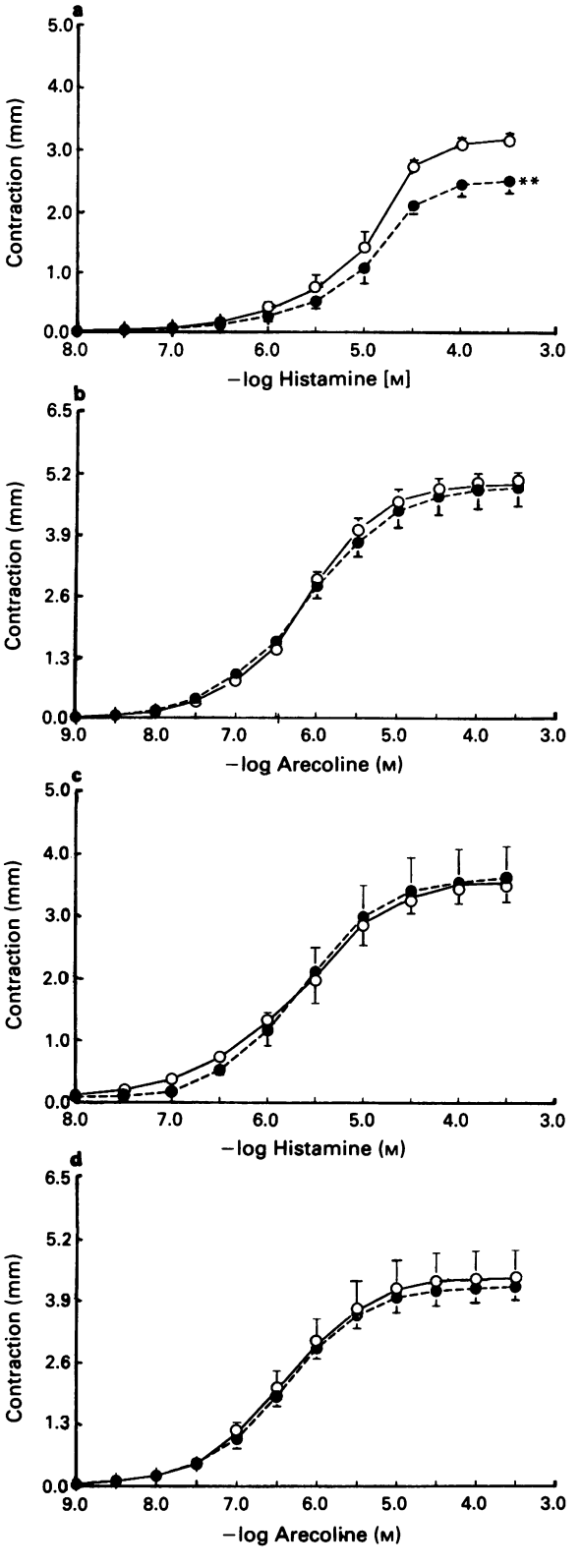

Figure 2 Concentration-response curves for histamine (a and c) and arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreatment with oxatomide (2.5 mg kg^{-1} b.wt., a and b) or nedocromil (2.5 mg kg^{-1} b.wt., c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. Oxatomide and nedocromil inhibited the increased tracheal contraction in response to histamine and arecoline in the PI-3 virus-treated groups.

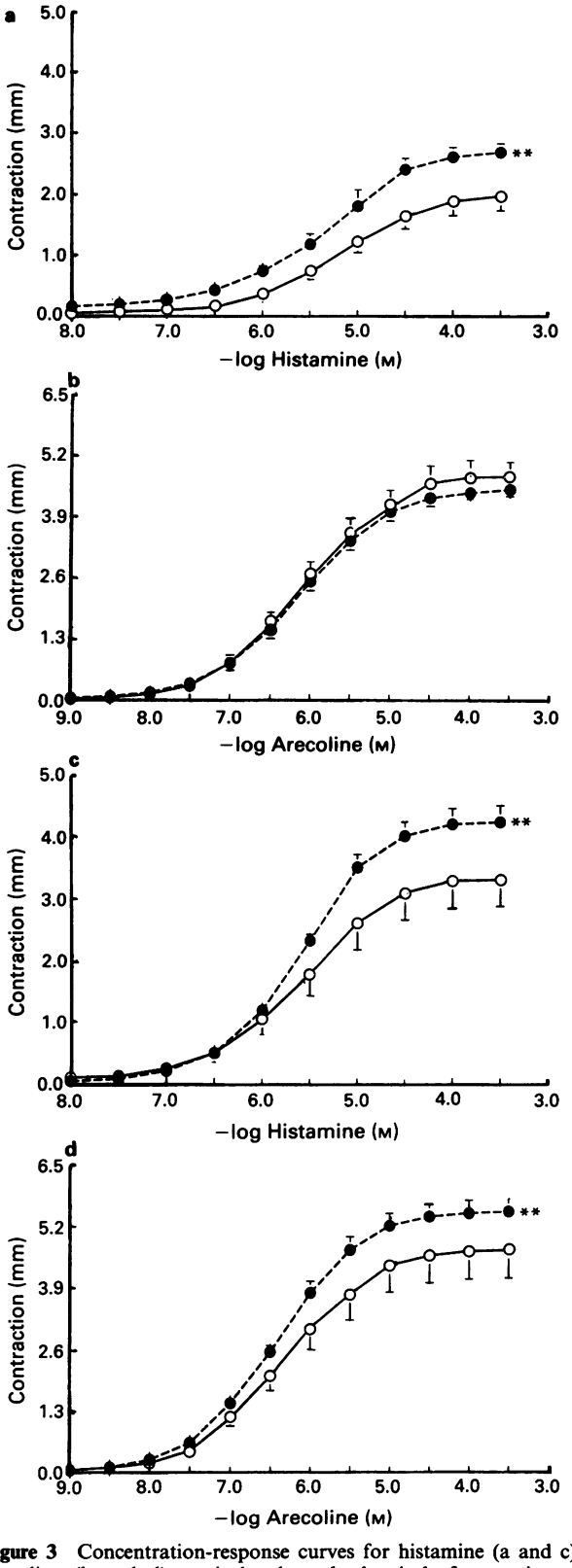

Figure 3 Concentration-response curves for histamine (a and c) or arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreatment with flunarizine (2.5 mg kg^{-1} b.wt., a and b) or ketanserin (0.63 mg kg^{-1} b.wt., c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. The drugs did not affect the virus-induced airway hyperresponsiveness to histamine. The C/R curves were significantly shifted upwards (**$P<0.01$, two way ANOVA) in the PI-3 virus groups when compared to the control group. Flunarizine (2.5 mg kg^{-1} b.wt.) suppressed the histamine C/R curve in both the group inoculated with control solution and that inoculated with PI-3 virus (a). Flunarizine inhibited the virus-induced tracheal hyperresponsiveness to arecoline (b).

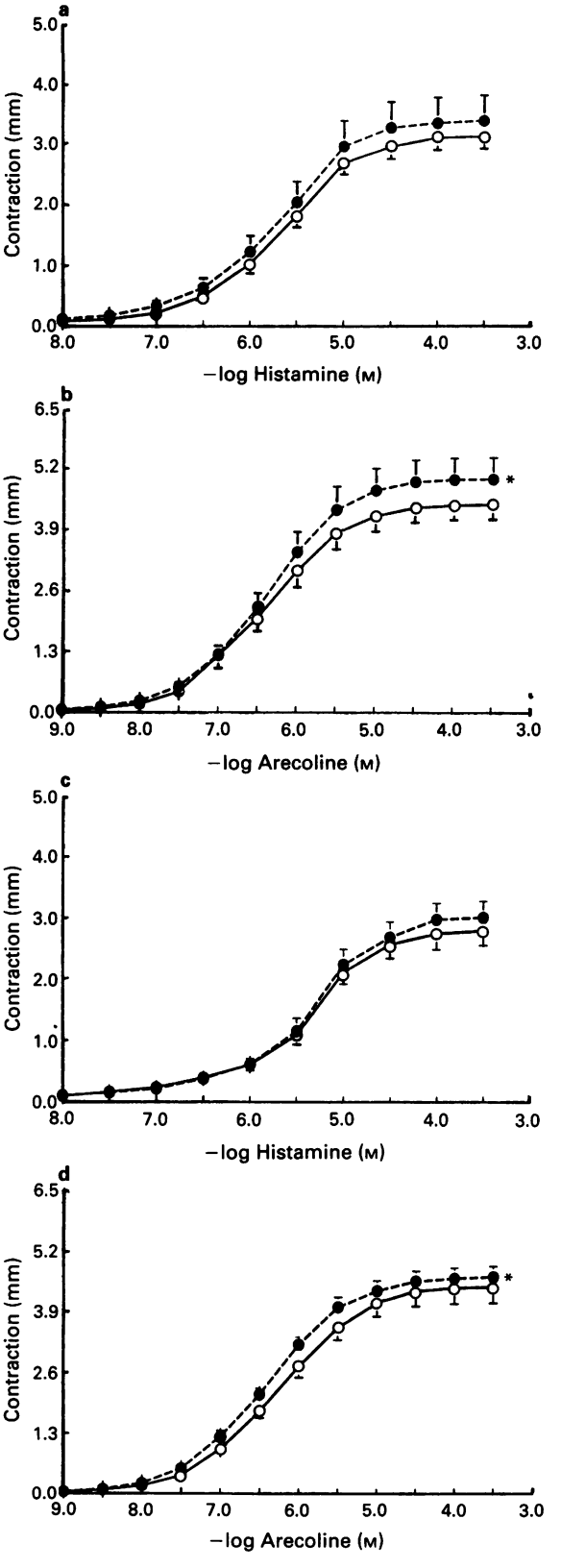

Figure 4 Concentration-response curves to histamine (a and c) and arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreated with WEB 2170 (10 mg kg^{-1} b.wt., a and b) or levocabastine (0.25 mg kg^{-1} b.wt., c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. WEB 2170 and levocabastine inhibited the increased airway contraction in response to histamine in the PI-3 virus-treated groups. The arecoline C/R curves were still slightly enhanced in the PI-3 virus group after pretreatment with WEB 2170 or levocabastine ($P<0.05$, two way ANOVA).

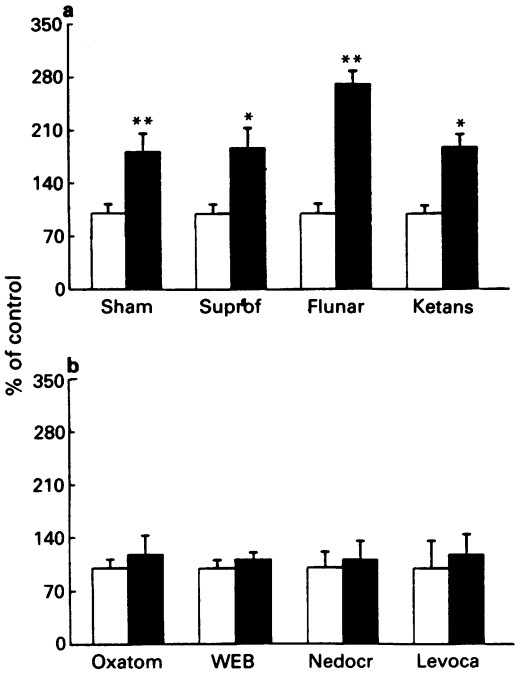

Figure 5 Broncho-alveolar cells in lung lavage fluid from guinea-pigs, inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes (a). Solvent solution (1.0 ml kg^{-1}, b.wt.), suprofen (Suprof. 5.0 mg kg^{-1}, b.wt.), flunarizine (Flunar 2.5 mg kg^{-1}, b.wt.), ketanserin (Ketans, 0.63 mg kg^{-1}, b.wt.); (b) oxatomide (Oxatom, 2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (Nedocr, 2.5 mg kg^{-1}, b.wt.), levocabastine (Levoca, 0.25 mg kg^{-1}, b.wt.). The data are expressed as percentage of the mean number of broncho-alveolar cells of each respective control group. The increase in the number of broncho-alveolar cells induced by PI-3 virus is inhibited by oxatomide, WEB 2170, nedocromil, and levocabastine. $*P<0.05$, $**P<0.01$ Student's t test (unpaired); $n = 5-8$. The relative numbers of the individual cell types are presented in Table 3.

isolated from animals inoculated with control solution or PI-3 virus and medicated with WEB 2170 or levocabastine did not differ significantly (Figure 4a and c), arecoline C/R curves in medicated animals remained slightly shifted upward ($P<0.05$, ANOVA). The EC_{50} value for both agonists did not differ between preparations obtained from virus-infected animals relative to those from guinea-pigs inoculated with control solution (Tables 1 and 2).

There were no differences in the slope factors of the histamine or arecoline C/R curves between tracheae obtained from animals inoculated with control solution or PI-3 virus, irrespective of the pharmacological probes used (Tables 1 and 2).

Number and types of broncho-alveolar cells

The absolute number of broncho-alveolar cells in the lung lavage fluid in the control animals was $14.5 \pm 1.8 \times 10^6$ cells. This number was almost doubled, to $26.3 \pm 3.5 \times 10^6$, in the virus-treated guinea-pigs. The increase in the number of broncho-alveolar cells was due to an increase in the absolute number of cell types: alveolar macrophages 1.5 fold. monocytes 3.6 fold, lymphocytes 20.0 fold, eosinophils 3.9 fold and neutrophils 3.6 fold.

Suprofen, flunarizine or ketanserin pretreatment did not modify the increase in the number of broncho-alveolar cells in the lung lavage fluid of guinea-pigs inoculated with PI-3 virus (Figure 5a). However, oxatomide, WEB 2170, nedocromil, and levocabastine which inhibited the virus-induced tracheal hyperresponsiveness, also inhibited the increase in the number of broncho-alveolar cells (Figure 5b).

The inhibition of the increase in *absolute* number of broncho-alveolar cells in the virus-treated animals by oxatomide, WEB 2170, nedocromil, and levocabastine was mainly due to a reduction in the *absolute* number of alveolar macrophages.

In the virally-infected group, the relative number of the alveolar macrophages was decreased ($P<0.01$), while that of all other cell types significantly increased. Changes induced in

Table 3 Mean distribution of broncho-alveolar cells as % of total in guinea-pigs pretreated with pharmacological probes and inoculated with control solution or PI-3 virus

Treatment	*Macrophages*	*Monocytes*	*Lymphocytes*	*Eosinophils*	*Neutrophils*
Sham					
Control	88.3 ± 2.10‡	0.63 ± 0.26	0.25 ± 0.16	7.54 ± 1.45	3.25 ± 1.15
PI-3	71.4 ± 1.81§§	1.71 ± 0.36§	3.71 ± 1.02§§	15.5 ± 0.16§§	7.71 ± 1.41§
Suprofen					
Control	89.7 ± 2.80	1.00 ± 0.45	0.16 ± 0.16	4.17 ± 0.75	5.52 ± 2.97
PI-3	74.3 ± 3.79§§	1.83 ± 0.54	1.33 ± 0.21§	17.3 ± 3.00§§	5.17 ± 1.08
Flunarizine					
Control	88.7 ± 2.50	0.33 ± 0.33	0.33 ± 0.21	4.67 ± 0.99	6.00 ± 1.98
PI-3	75.8 ± 4.15§	2.00 ± 0.63§	2.50 ± 0.56§§	10.7 ± 2.16§	9.31 ± 1.60
Ketanserin					
Control	87.6 ± 1.10	3.43 ± 1.22	0.60 ± 0.40	4.62 ± 0.98	4.62 ± 0.98
PI-3	74.2 ± 5.29§	4.58 ± 0.92	5.40 ± 2.44	9.78 ± 2.77	5.98 ± 3.22
Oxatomide					
Control	80.2 ± 3.51	2.36 ± 0.37	1.75 ± 0.62	8.30 ± 1.08	7.40 ± 2.96
PI-3	64.7 ± 4.77§	2.71 ± 0.50	6.06 ± 0.88§§	12.4 ± 2.21	14.3 ± 3.98
WEB 2170					
Control	83.4 ± 3.30	1.52 ± 0.57	0.00 ± 0.00	9.38 ± 0.63	6.50 ± 2.59
PI-3	74.3 ± 4.06	1.17 ± 0.31	3.33 ± 0.67§§	16.2 ± 2.17§	4.67 ± 1.71
Nedocromil					
Control	79.9 ± 2.30	1.42 ± 0.84	0.86 ± 0.40	10.1 ± 1.01	7.57 ± 1.65
PI-3	75.0 ± 5.00	1.17 ± 0.52	4.13 ± 2.08	16.3 ± 3.53	2.33 ± 0.49§
Levocabastine					
Control	87.0 ± 5.27	1.51 ± 0.56	0.83 ± 0.47	5.50 ± 2.70	5.17 ± 2.29
PI-3	76.0 ± 3.80	2.17 ± 0.70	3.00 ± 0.93	11.2 ± 3.26	7.50 ± 1.90

‡ Results are expressed as means ± s.e.mean.
§ $P<0.05$; §§$P<0.01$ as compared with the control group.
$n = 5-8$.

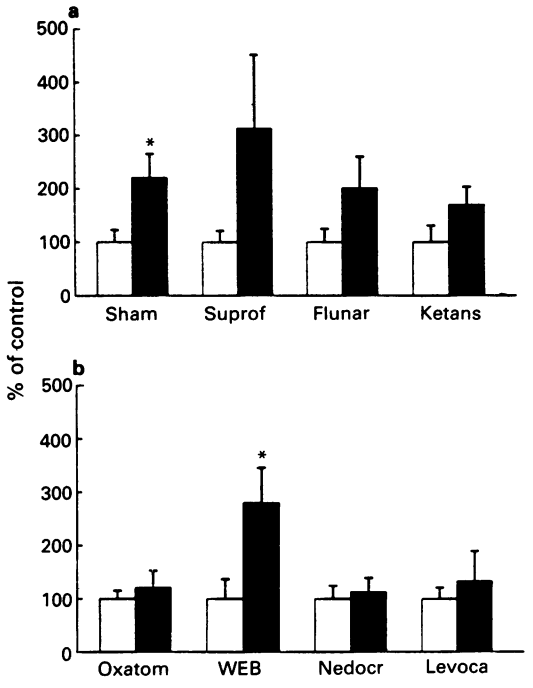

Figure 6 Albumin content in lung lavage fluid from guinea-pigs, inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes. (a) Solvent solution (1.0 ml kg^{-1} b.wt.), suprofen (5.0 mg kg^{-1}, b.wt.), flunarizine (2.5 mg kg^{-1} b.wt.), ketanserin (0.63 mg kg^{-1} b.wt.); (b) oxatomide (2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (2.5 mg kg^{-1}, b.wt.), levocabastine (0.25 mg kg^{-1}, b.wt.). Abbreviations as in Figure 5. Data (means ± s.e.mean) are expressed as percentage of the mean albumin concentration of each respective control group. The increase in albumin in lung lavage fluid induced by PI-3 virus is reduced by oxatomide, nedocromil, and levocabastine. $*P<0.05$, Student's t test (unpaired); $n = 5-8$.

the relative number of broncho-alveolar cells by pretreatment with the pharmacological probes were not directly associated with changes in airway reactivity (Table 3).

Albumin content in lung lavage fluid

In virally-infected animals, the albumin concentration in the broncho-alveolar lavage fluid was significantly higher (42.5 ± 8.5 μg ml^{-1}; $P<0.05$, $n = 8$) in comparison with that of guinea-pigs inoculated with control solution (19.2 ± 4.4 μg ml^{-1}; $n = 7$). After pretreatment with suprofen, ketanserin, or flunarizine, the albumin content was increased, although not significantly, in the PI-3 virus groups by 210%, 100% and 70%, as compared with the respective control groups (Figure 6a). PAF-receptor antagonism (WEB 2170) during the development phase of the virally-induced tracheal hyperresponsiveness did not modify the increase of albumin into the broncho-alveolar lavage fluid (Figure 6b, $P<0.05$). By contrast, treatment with either oxatomide, nedocromil or levocabastine abolished such an increased leakage of albumin (percentage increase: 20%, 13% and 33%, respectively; Figure 6b).

Chemiluminescence generation by broncho-alveolar cells

Basal chemiluminescence generation (i.e. stimulated with 200 μl control solution) by broncho-alveolar cells obtained from animals inoculated with control solution or PI-3 virus did not differ between the two groups, irrespective of the pretreatment. In the sham groups, the basal chemiluminescence production by broncho-alveolar cells obtained from animals inoculated with control solution was 148.9 ± 27.4 mV ($n = 9$) and with virus was 138.2 ± 24.1 ($n = 9$). H_1-receptor blockade (levocabastine) reduced the basal chemiluminescence production in both inoculated groups (control solution, 69.09 ± 9.20 mV; $n = 6$ vs virus, 88.70 ± 17.7 mV; $n = 6$). Such a decrease was also found after treatment with WEB 2170 (control solution, 93.20 ± 7.10 mV; $n = 6$ vs virus, 83.40 ± 8.50 mV; $n = 6$). The other drugs did not affect the basal chemiluminescence generation (data not shown). In the group inoculated with control solution, the addition of PI-3 virus to broncho-alveolar cells increased the chemiluminescence production to 1284.6 ± 130 mV; this increase was significantly less ($P<0.05$) in the animals infected with PI-3 virus (742.6 ± 164.4 mV). Suprofen, flunarizine and ketanserin did not abolish this diminution (Figure 7a). The chemiluminescence production after PI-3 virus stimulation was decreased in both groups after treatment with flunarizine, but the difference between the group inoculated with control solution and that with PI-3 virus remained (774.4 ± 125.0 mV vs 230.2 ± 43.8 mV, $P<0.05$). Pretreatment with oxatomide, WEB 2170, nedocromil and levocabastine did not modify this decrease either (Figure 7b). After stimulation of the broncho-alveolar cells obtained from virus-infected animals, with PI-3 the chemiluminescence was still, although not significantly, decreased as compared to the respective control groups.

The increase in chemiluminescence generation by broncho-alveolar cells after stimulation with zymosan was significantly diminished in the virally-infected group (control solution, 1176.7 ± 110.8 vs virus, 838.2 ± 46.1 mV, $P<0.05$, Figure 8a). Again, in the groups treated with flunarizine this stimulation

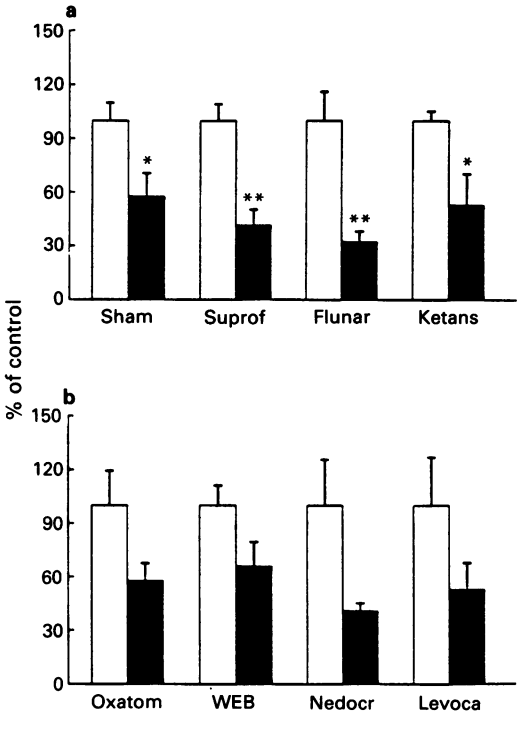

Figure 7 PI-3 virus-induced chemiluminescence generation by broncho-alveolar cells obtained from guinea-pigs inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes. (a) Solvent solution (1.0 mg kg^{-1}, b.wt.), suprofen (5.0 mg kg^{-1}, b.wt.), flunarizine (2.5 mg kg^{-1}, b.wt.), or ketanserin (0.63 mg kg^{-1}, b.wt.). (b) Oxatomide (2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (2.5 mg kg^{-1}, b.wt.), levocabastine (0.25 mg kg^{-1} b.wt.). Abbreviations as in Figure 5. The data are expressed as percentage of the mean peak chemiluminescence of each respective control group. The diminished chemiluminescence production by broncho-alveolar cells from animals treated with PI-3 virus was not affected by any pretreatment with drugs. $*P<0.05$; $**P<0.01$, Student's t test (unpaired); $n = 5-8$.

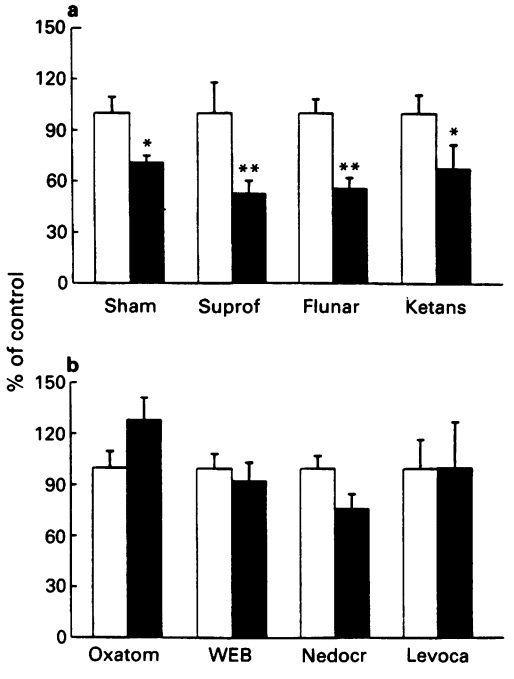

Figure 8 Zymosan (2.5 mg)-induced chemiluminescence generation by broncho-alveolar cells obtained from guinea-pigs inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes. (a) Solvent solution (1.0 ml kg^{-1}, b.wt.), suprofen (5.0 mg kg^{-1}, b.wt.), flunarizine (2.5 mg kg^{-1}, b.wt.), or ketanserin (0.63 mg kg^{-1} b.wt.). (b) Oxatomide (2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (2.5 mg kg^{-1}, b.wt.), levocabastine (0.25 mg kg^{-1}, b.wt.). Abbreviations as in Figure 5. The data are expressed as percentage of the mean peak chemiluminescence of every respective control group. The diminished chemiluminescence production by broncho-alveolar cells from animals treated with PI-3 virus was inhibited by oxatomide, WEB 2170, nedocromil, and levocabastine. $*P<0.05$; $**P<0.01$, Student's t test (unpaired); $n = 5-8$.

was less pronounced, but the difference remained (control solution, 764.8 ± 62.5 mV vs virus, 427.3 ± 47.2 mV, $P<0.05$). Suprofen and ketanserin did not affect the virus-induced reduction in chemiluminescence generation after stimulation with zymosan (Figure 8a). However, oxatomide, WEB 2170, levocabastine, and to a lesser extent nedocromil, attenuated the reduced chemiluminescence generation by broncho-alveolar cells obtained from virally-infected animals (Figure 8b).

Histamine nebulization

The histamine C/R curve on tissues obtained from histamine nebulized guinea-pigs ($n = 6$) was significantly ($P<0.01$, ANOVA) shifted downwards as compared with the tracheae obtained from saline-treated animals ($n = 6$, data not shown). The maximal response was decreased, although not significantly, by 20%. In contrast, the arecoline C/R did not differ between the saline and histamine nebulized group. In addition, the EC_{50} values and the slope factors did not differ significantly between both experimental groups after the histamine or arecoline C/R curves.

The total number of inflammatory cells isolated from the airways of animals nebulized with saline was $13.0 \pm 1.4 \times 10^6$ and with histamine $9.0 \pm 1.3 \times 10^6$ ($P>0.05$). The chemiluminescence production after stimulation with zymosan did not differ also between the saline-nebulized group (822.6 ± 159 mV) and the histamine-nebulized group (835.1 ± 47.0 mV).

Discussion

This study shows that virus-induced airway hyperresponsiveness in the guinea-pig is inhibited by drugs interfering with histamine release or with the binding of histamine to its receptor. Oxatomide and nedocromil have mast cell stabilizing effects (Awouters *et al.*, 1979; De Clerck *et al.*, 1981; Auty, 1986). In addition, oxatomide as well as levocabastine, blocks H_1-histamine receptor binding sites (Awouters *et al.*, 1979; De Clerck *et al.*, 1981; Van Wauwe, 1989). Histamine increases vascular permeability and has chemotactic activities (Clarck *et al.*, 1975; Hill, 1990). Indeed, oxatomide, nedocromil, and levocabastine inhibit the virus-induced increase in the number of inflammatory cells and the concentration of albumin in lung lavage fluid. In addition, these drugs inhibited the virus-induced decrease in chemiluminescence production after stimulation with zymosan. Interestingly, the drugs that did not modify the virus-induced tracheal hyperresponsiveness to histamine i.e. the cyclo-oxygenase inhibitor, suprofen, the 5-HT_2 receptor antagonist, ketanserin, and the Ca^{2+} overload blocker, flunarizine, did not modify the influx of inflammatory cells, the increase in albumin, or the decrease in chemiluminescence production after stimulation with zymosan. Flunarizine inhibited the histamine-induced contraction and shifted the C/R curve to the right; but the virus-induced airway hyperresponsiveness to histamine remained. The shape of the arecoline C/R curve was not changed as compared to the sham-treated group, but the virus-induced increased airway contraction was abolished. Histamine needs extracellular Ca^{2+} to elicit a contraction of the trachea, and arecoline increases the intracellular Ca^{2+} concentration via Ca^{2+} release from intracellular stores. This difference might offer a solution for the discrepant results.

The PAF antagonist, WEB 2170, was an exception to the rule in the way that this compound inhibited the increased airway responsiveness and the influx of inflammatory cells, and abolished the decrease in chemiluminescence production in response to zymosan, but did not affect the virus-induced increase of albumin in the lung lavage fluid. Therefore, an increase in albumin leakage may contribute, but is not primarily responsible for the airway hyperresponsiveness.

Basal chemiluminescence production of the broncho-alveolar cells did not differ between the groups inoculated with control solution and PI-3 virus in all pretreated animals. PI-3 virus was a very potent stimulus for broncho-alveolar cells as demonstrated by a marked increase in chemiluminescence production *in vitro*. The chemiluminescence production by broncho-alveolar cells isolated from virally-infected animals was reduced when stimulated with PI-3 virus. None of the pharmacological agents used could abolish this diminution. In contrast, the reduced chemiluminescence production found after stimulation with zymosan was inhibited by oxatomide, nedocromil, WEB 2170, and levocabastine; drugs that also inhibited the influx of inflammatory cells. It may be suggested that on the one hand the activity of broncho-alveolar cells is diminished in a non-specific way (for zymosan), and on the other hand the activity is diminished in a specific way (for PI-3 virus) since none of the drugs used, could inhibit the decreased chemiluminescence upon additional PI-3 virus stimulation. The possibility cannot be excluded that the increase in inflammatory cell numbers will result in a diminished activation of the cells, for instance by local changes in pH and/or release of reactive oxygen radicals and lysosomal enzymes. Therefore, the inhibition of the cell influx by oxatomide, nedocromil, WEB 2170, and levocabastine may prevent excessive release of mediator and enzyme release by inflammatory cells and subsequently inhibit the diminished activity of broncho-alveolar cells.

Many studies have suggested a role for inflammatory cells in the induction of airway hyperresponsiveness (Barnes *et al.*, 1988; Djukanovic *et al.*, 1990). Indeed, in the present study an association was found between the influx of inflammatory cells and the occurrence of airway hyperresponsiveness.

Drugs, such as oxatomide, nedocromil, WEB 2170, and levocabastine, that reduced the influx of cells, especially alveolar macrophages, also inhibited the increase in tracheal contractions. In our laboratory supporting evidence was found that inflammatory cells can modify airway responsiveness. Tracheae obtained from healthy non-treated guinea-pigs incubated *in vitro* with broncho-alveolar cells obtained from virus-infected animals, demonstrated an increased contraction in response to histamine and arecoline (Folkerts *et al.*, 1992c).

PAF and histamine can increase vascular permeability and can attract inflammatory cells into the respiratory tract (Clark *et al.*, 1975; Tate *et al.*, 1982; Lellouch-Tubiana *et al.*, 1988; O'Donnell & Barnett, 1987; Hill, 1990). PAF is one of the major products formed by alveolar macrophages. Histamine can be released by metachromatic cells after stimulation with: histamine releasing factors and interferons (Lett-Brown *et al.*, 1989), reactive oxygen radicals (Mannaioni & Masini, 1988) or PAF (Okuda *et al.*, 1988). These products are likely to be released during viral infections (Hall *et al.*, 1978). Recently we obtained evidence, that in the respiratory tract of virus-infected animals the mast cell morphology and number was changed and that the histamine concentration was doubled in the broncho-alveolar lavage fluid (Folkerts, 1993). Similar findings were published by others. Basal and stimulated histamine release from mast cells isolated from the airways of calves 7 days after inoculation with PI-3 virus, is significantly increased (Ogunbiyi *et al.*, 1988). Moreover, basophils isolated from guinea-pigs 4 days after PI-3 virus infection released more histamine after stimulation, than did basophils from control animals (Graziano *et al.*, 1989).

In patients infected with respiratory syncytial virus the release of histamine in nasopharyngeal secretions was detected significantly more often and in higher concentrations in patients with wheezing (Welliver *et al.*, 1981). In addition, in asthmatic patients positive correlations were found between the degree of bronchial hyperresponsiveness and (a) mast cell histamine release (Wardlaw *et al.*, 1988), (b) the number of mast cells in BAL fluid (Djukanovic *et al.*, 1990) and (c) the concentration of histamine in BAL fluid (Casale *et al.*, 1987). Interestingly, treatment of mild asthmatics with a specific non-sedating H_1-receptor blocker reduced asthmatic symptoms such as cough, wheeze, chest tightness, breathlessness, and inhibited the development of airway hyperresponsiveness (Rafferty, 1990). In contrast, however, nebulization of histamine for 4 successive days did not result in airway hyperresponsiveness, but instead, to histamine tachyphylaxis. Moreover, the number and activity of the broncho-alveolar cells was also not changed. These results support the idea that inflammatory cells/mediators are required in addition, to modify airway responses.

From literature data and the results presented in this study some suggestions can be made as to how viral respiratory infections cause airway hyperresponsiveness. Inflammatory cells seem to play an important role. Histamine and PAF, not only increase vascular permeability, but also attract inflammatory cells into the lungs. The expression of adhesion molecules on endothelial cells induced by histamine (Pober & Cotran, 1990) and on airway epithelial cells induced by viruses (Tosi *et al.*, 1992), probably potentiates the transport of inflammatory cells into the lumen of the airways. The inflammatory cells release an array of mediators that probably modify airway responses. The epithelial damage observed after a viral infection (Folkerts *et al.*, 1992a) may be caused by products released from these inflammatory cells and/or by the growth of viruses in epithelial cells. The loss of barrier function together with a decreased formation of an epithelium-derived relaxing factor(s) can also influence the contractility of the tracheae. Finally, epithelial damage exposes sensory nerve endings that can be stimulated by histamine and other mediators, leading to a release of neuropeptides (Barnes *et al.*, 1990; Maggi, 1991). Airway contractions in response to substance P and capsaicin are indeed increased in PI-3 virus-infected guinea-pigs (Saban *et al.*, 1987). Further, as shown by Jacoby *et al.* (1988), the enzymatic activity of enkephalinase is decreased and the reactivity to substance P is increased in ferret airway tissues incubated with influenza virus for 4 days. It cannot be excluded that an enhanced concentration of neuropeptides in the respiratory tract influences the contractility to histamine and arecoline.

It is concluded that histamine is probably involved in a cascade of events leading to airway hyperresponsiveness after a viral infection. It must be stressed that histamine is not solely responsible for the observed effects, and that inflammatory cells/mediators and epithelial damage are likely to be additional factors that contribute to the increased tracheal responsiveness.

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