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Effect of experimental airway inflammation on bronchial hyper-responsiveness induced by Broncho-Vaxom in dogs

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We studied correlation between airway hyper-responsiveness induced by local exposure to a macrophage inductor Broncho-Vaxom and the development of airway inflammation in dogs. To detect airway inflammation, bronchoalveolar lavage and biopsy of airway mucosa were performed. The airway responsiveness was registered by capnograph measuring gas-exchange disturbances during obstructive reactions provocated by inhalation of various concentrations of acetylcholine aerosol. Broncho-Vaxom generated a protracted airway inflammation characterized by a slight and reversible increase in the number of neutrophils at 24 h after induction, and by a long-lasting influx of macrophages peaked about at the second week. The number of macrophages turned to initial levels 3 weeks later. Macrophages migrating to the bronchoalveolar surface were activated because peroxidase positivity and bearing C_{3b} receptors of these cells increased gradually during the inflammatory process. Airway responsiveness measured at 3, 6 and 24 h after induction did not differ significantly from baseline values, but hyper-responsiveness was developed at 96 h using 0.5 and 1.0% acetylcholine aerosol ($p < 0.01$ and $p < 0.001$) during the non-purulent, macrophage-mediated inflammation. This situation modelled by Broncho-Vaxom induction is very similar to those observed in children with recurrent obstructive bronchitis. The results suggest that a macrophage-mediated inflammation caused by antigens, infections or pollutants may generate a long-lasting airway hyper-responsiveness.

Bronchial reactivity, a characteristic feature of asthma is defined as increased sensitivity of the airways to physical, chemical, and pharmacologic stimuli [1]. Although the precise causes of hyperreactivity are uncertain, recently airway inflammation has been taken into consideration as an important cause of increased levels of bronchial responsiveness [2]. So far, a series of studies has examined types of cells and mediators responsible for ozone-induced changes in airway reactivity [3, 4, 5, 6]. Increases in responsiveness after ozone are followed by enumeration of neutrophils and airway epithelial cells recovered in bronchoalveolar lavage (BAL) fluid [3]. Because of the increase in bronchial reactivity generated by ozone lasts only for few days, changes in the airways may imperfectly mimic the abnormalities of asthma [7]. This difference has led the investigators to speculate that a type of cell other than the neutrophil is responsible for the hyper-reactivity [2].

Recently, children with severe epi-

sodes of recurrent obstructive bronchitis have been investigated by BAL to reveal features of the chronic mucosal inflammation observed bronchoscopically [8, 9]. A striking influx of macrophages was registered in the symptom-free periods without accumulation of neutrophils. This airway inflammation may be induced by respiratory infections, as well as by antigens, environmental allergens and pollutants. It is presumable that, once initiated, inflammation should become a self-perpetuating process in our patients, and it should be responsible for airway hyper-responsiveness [10].

We undertook the present study to determine whether the airway hyper-responsiveness induced by local exposure to a macrophage inductor, Broncho-Vaxom correlated with the development of airway inflammation in dogs. To detect airway inflammation, we performed biopsies of the airway mucosa as well as BAL, and the lavagable cells were characterized. To investigate airway responsiveness, gas-exchange disturbances were measured by capnograph during obstructive episodes generated by acetylcholine stimuli. The dogs were used as their own control for the development of inflammation and hyper-responsiveness.

Materials and Methods

Animals

12 normal adult dogs of both sexes, weighing 5 to 15 kg, were used. On Day 1, each dog was first lightly anaesthetized intravenously with hexobarbital-Na (10 mg/kg), secured in the supine position.

Blood

Blood was obtained by puncture of the femoral vein for total and differential leukocyte counts.

Airway responsiveness

Then, dogs were intubated, and changes in the CO_2 contents of exhaled air were registered continuously by capnograph (Jägers' type, Würzburg, FRG). Capnography is suitable for a quantitative estimation for the response to acetylcholine provocation because obstructive processes are characterized by an uneven distribution of air [11, 12, 13]. Bypass-flow sampling was done at an aspiration rate of 0.5 l/min through a soft plastic tube inserted to a depth of 3 cm in the endotracheal tube. All tubes used in the experiments were measured 30 cm in length and 3 mm in diameter. Airway responsiveness was assessed beginning 30 min after induction of anaesthesia by obtaining a dose-response curve to acetylcholine aerosol. After detecting the baseline values, isotonic, sterile sodium chloride solution was delivered to the airways for 30 s (5 breaths); next, CO_2 content of expirated air was registered for 3 min. If the result showed variable breathing, the values of several expirations were averaged. This process was repeated every 4 min for each of increasing doses of acetylcholine. The dose of acetylcholine was increased: 0.1% to 1.0%. Acetylcholine aerosols were generated from a jet-type nebulizer (Glück's type model, Medicor Works, Hungary) and delivered to the dog's airways via the endotracheal tube at a constant air flow (0.1 l/s). The output of the nebulizer was 0.5 ml/min.

Evaluation of capnograms

Practical evaluation of the capnograms was described elsewhere [11, 12]. Briefly, all capnograms show an exponential seg-

ment which passes into and terminates with a linear province (plateau). The first indicates the interproportion between the air of the dead space and the alveolar air, the second reveals the concentration of CO_2 in alveolar air. Capnograms are characterized by the quotient of two values: $\frac{\text{tg}\,\alpha}{\text{tg}\,\beta}$ where α and β mean angles formed the exponential segment and the linear plateau with the axis t. In this study, the variation of data of capnograms was expressed in the relative per cent of the baseline values.

Biopsy of airway mucosa

After removal of the endotracheal tube a rigid tube bronchoscope (8 mm in diameter) (Friedel's Bronchoscope model VEB MLW Medizinische Geräte, Berlin, GDR) was inserted into the trachea and a biopsy was taken from the major carina using Friedel's forceps. The biopsy specimen was placed immediately into 10% buffered formalin and later was embedded in paraffin to cutting and staine dwith haematoxylin-eosin.

BAL procedure

Lavage was performed using 3×20 ml sterile, isotonic sodium cholide solution at 37 °C through a plastic catheter wedged in the bronchus of the lower lobe. The fluid was immediately aspirated. The average recovered volume was 73.6% of the instillated fluid. The lavage procedure was tolerated with no mortality and apparent morbidity. Mucus strands in the recovered fluid were removed by filtration through several layers of very loose cotton gauze, and cells collected by centrifugation (800*g* for 10 min). The supernatants were immediately frozen at −40 °C for further investigations. Cells were resuspended in Hank's balanced salt solution (HBSS), and cell count as well as differential cell count on a Giemsa stained smear were determined.

Cytochemistry

Esterase-1 activity was investigated according to Ornstein [14]. Peroxidase activity was determined according to Kaplow [15].

Cell surface receptors

The presence of Fcγ and C_{3b} receptors was detected on glass-adherent cells with rabbit IgG-coated sheep red blood cells (SRBC) and by the use of SRBCs coated with IgM fraction of rabbit anti SRBC serum and complement from fresh mouse serum [16].

Airway inflammation

Bronchoalveolar inflammation was generated by instillation of 7 mg Broncho-Vaxom (OM Laboratories, Geneva, Switzerland and Biogal Works, Debrecen, Hungary) in 5 ml isotonic sodium chloride solution into the right main bronchus. The insoluble components of this medicine were removed by centrifugation (1000*g* for 10 min). Broncho-Vaxom is a bacterial lysate without antigenic features whose immunostimulatory activity has been demonstrated both pharmacologically and chemically [17]. This lysate of eight bacteria (H. influenzae, D. pneumoniae, Klebsiella pneumoniae and ozaneae, S. aureus, S. pyogenes and viridans, N. catarrhalis) was expected as a potent alveolar macrophage activator and as an inductor of a non-purulent inflammation on the bronchoalveolar surface.

At intervals between 3, 6 and 24 h after induction, the same procedure mentioned and detailed above was used in pairs of dogs to avoid the relavaging in the same animals within 72 h. Since the course of relavaging could produce striking increase in the percentage of neutrophils in the lavage fluid [18]. For the same reason, the baseline parameters were reg-

istered a few days (6—12 days) before the Broncho-Vaxom induction. All dogs, however, were investigated at 96 h using the whole procedure.

Statistical evaluations

For the statistical evaluation of the differences use was made of Student's *t*-test. The standard errors of the means (SEM) were calculated for each group.

Results

Blood leukocytes

The mean values for leukocyte counts, total and differential revealed a marked increase in the total cell count and in number of neutrophils in venous blood at 24 h after induction, but they returned almost to the initial values by 96 h (Fig. 1). No evidence of lymphocytosis and eosinophilia or other haematological abnormality could be found.

Biopsy of airway mucosa

Airway biopsy sections obtained from different sites of the major carina showed an increase in the number of neutrophils in the airway mucosa at 3 and 6 h after induction of Broncho-Vaxom. This moderate purulent reaction of the mucosa decreased gradually and the influx of mononuclear cells became dominant at 96 h after induction. So, the proportion of neutrophils and mononuclear cells infiltrated the mucosa was 1 : 4 at 96 h.

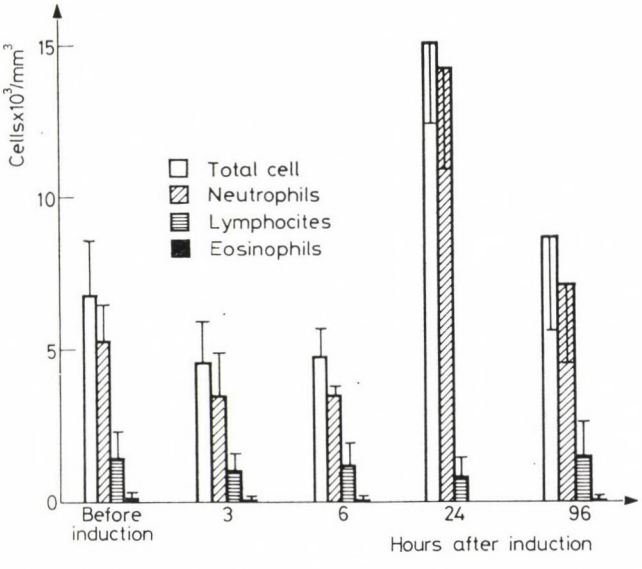


Fig. 1. Effect of Broncho-Vaxom on the number of types of cells in the venous blood after total number of cells is shown in consequence of the enumeration of neutrophils. Each bar represents the mean and SEM

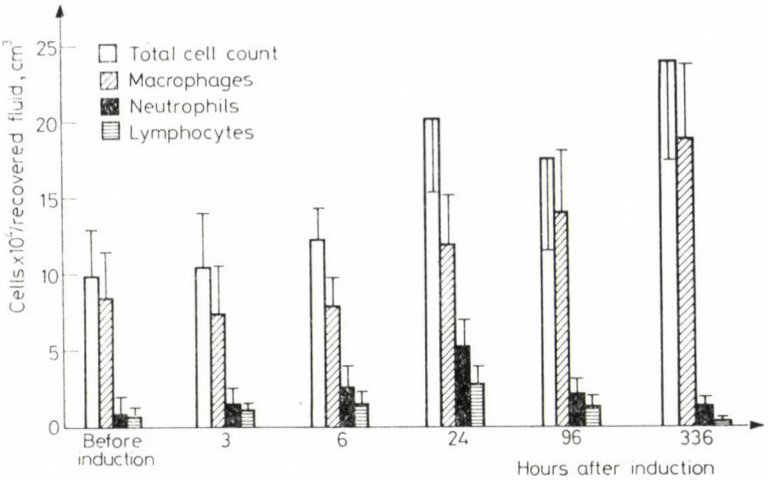


FIG. 2. Changes in the cell contents of the recovered lavage fluid samples after Broncho-Vaxom induction. The total cell yield and number of macrophages were increased significantly while the accumulation of neutrophils proved to be reversible. The slight increase in the number of lymphocytes was considered as a result of increased permeability of the mucosa during the phase of inflammation characterized by the transient influx of neutrophils. Each bar represents the mean and SEM

Yield of cells

The total number of cells isolated from the lavage fluid rose significantly within 24 h after instillation of Broncho-Vaxom (Fig. 2), and diminished temporarily at 96 h and then showed a rise to maximal levels at 14 days. The total cell count decreased to a near-normal level at 21 days after induction (data not shown). In these suspensions, the number of macrophages increased gradually during 2 weeks, and declined at later times to about the normal value at 21 days (data not shown). The number of neutrophils peaked at 24 h after instillation of Broncho-Vaxom and dropped rapidly to the initial level at 96 h. Similar changes in the number of lymphocytes could be observed.

Functional characteristics of macrophages

The number of peroxidase positive macrophages showed a moderate rise up to 96 h. Almost all macrophages were positive for esterase staining (Fig. 3). A great majority of alveolar macrophages carried Fc γ receptors, but the percentage of C_{3b} receptor-bearing cells increased slightly with the time after Broncho-Vaxom induction.

Airway responsiveness

Data registered during the provocation of dogs are shown in Fig. 4. All dogs reacted markedly to the inhalation of acetylcholine. The aver-

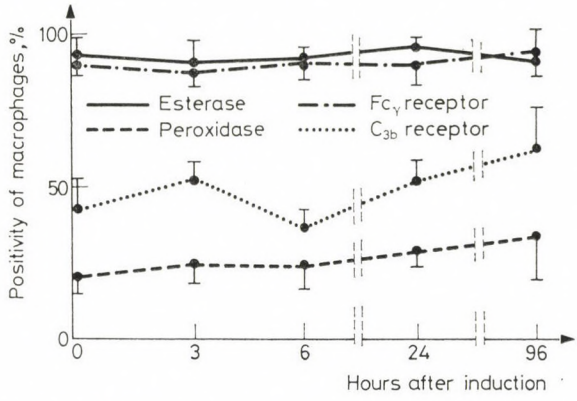
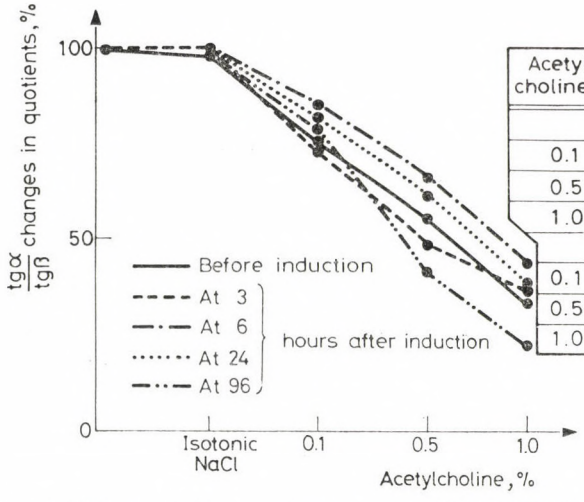


FIG. 3. Functional characteristics of alveolar macrophages after instillation of Broncho-Vaxom. Most of macrophages carried Fcγ receptors and were positive for esterase staining. A slight increase in the percentage of peroxidase positive macrophages and of bearing C_{3b} receptors was observed during the first four days after Broncho-Vaxom induction. Data points represent the mean and SEM values



Acetyl-choline, %	Before induction		
			at 96 hours
0.1	75.40±13.0	NS	79.70±19.2
0.5	54.60± 9.9	p>0.01	42.36±10.4
1.0	33.35± 7.4	p>0.001	23.76± 4.1
			at 24 hours
0.1	75.40±13.0	NS	81.60±13.6
0.5	54.60± 9.9	NS	62.20±24.0
1.0	33.35± 7.4	NS	38.85± 6.3

FIG. 4. Effect of inflammation generated by instillation of Broncho-Vaxom on the dose-response curve to acetylcholine aerosol in 12 dogs. The baseline values determined after each concentration of acetylcholine aerosol showed that each dog responded to the provocation. The responsiveness did not change significantly at 3, 6 and 24 h after induction of Bronco-Vaxom, but dogs became hyperresponsive at 96 h using 0.5 and 1.0% provocative concentrations of acetylcholine. Data points represent the average percentages of $\frac{tg\,\alpha}{tg\,\beta}$ quotients

age $\frac{tg\,\alpha}{tg\,\beta}$ quotients did not essentially differ from baseline values at 3, 6 and 24 h after instillation of Broncho-Vaxom. The decrease of values became pronounced at 96 h after inhalation of 0.5 and 1.0% solution of acetylcholine. These changes were statistically

significant compared to the baseline values indicating the increase in airway responsiveness during the later phase of inflammation characterized by the influx of alveolar macrophages.

Discussion

We generated a prolonged bronchoalveolar inflammation in dogs instillating Broncho-Vaxom into the right bronchial system. Apart from a transient neutrophilia in venous blood and a slight and reversible influx of neutrophils at 24 h on the bronchoalveolar surface, this inflammation could be considered as a non-purulent reaction characterized first of all by an increased influx of macrophages. The enumeration of macrophages lasted for about 3 weeks and the features of inflammation (at 96 h) were corresponded to those observed in children from 2 to 6 years of age with recurrent obstructive bronchitis [8]. These patients had suffered from serious episodes of obstructive bronchitis for months and airway hyper-responsiveness was detected. Allergic hyposensitization, focal infections, immunodeficient status, cystic fibrosis, malformations of heart- and large vessels, gastroesophageal reflux and other disorders which could be associated with obstructive symptoms were excluded previously. Clinical criteria of our patients referred to chronic bronchitis in childhood with recidive obstructive symptoms [19]. Signs of chronic inflammation of the mucosa were observed bronchoscopically. The extensive changes in the mucosa were appeared even though there was no recent history of an attack or a clinical symptom at least during the last weeks before the examination. A significant influx of macrophages was detected by BAL compared to those registered in young healthy volunteers by others and to our patients suffered from other chronic lung diseases [8]. This airway inflammation was considered to be a key determinant of the elevated airway responsiveness and a pathological basis of the frequent relapses [10].

Recently, investigators have turned to the use of experimental models of inflammatory changes in the airways associated with the induction of hightened bronchomotor responsiveness [2]. Airway inflammation caused by inhaled ozone was characterized by injury of airway epithelial cells and an influx of neutrophils [3]. After ozone exposure, however, the increase in bronchial responsiveness lasts only for a day or two [7]. The hyperreactivity in asthmatic patients and children with recurrent obstructive bronchitis persists for years, so the ozone-induced inflammation with neutrophil enumeration does not amount to the status produced by antigen challenges or viral infections.

In our experiments, the inflammation induced by Broncho-Vaxom was relatively long-lived. The stimulus of the lysate of bacterial walls proved to be intensive and resulted in the singnificant influx of macrophages. The morphological, cytochemical and functional characteristics of al-

veolar macrophages were like those of other tissue macrophages except for the peroxidase activity and the presence of C_{3b} receptors [16]. During the course of airway inflammation, the carrying of C_{3b} receptors and the peroxidase activity increased slowly that can be attributed to newly migrated monocytes into the lungs and to activation of resident macrophages [20].

We have demonstrated a progressive increase in the airway responsiveness correlated with the progressive increase in the number of macrophages on the bronchoalveolar surface at 96 h, but it was not so at 24 h after Broncho-Vaxom induction during the moderate influx of neutrophils. The role of airway inflammation in the development of hyper-responsive airways is complex and incompletely elucidated [21]. Respiratory infections are among the most common stimuli of asthmatic attacks. Viral infections can induce transient increase in airway responsiveness in non-asthamatics also [22]. In model, airway reactivity increased in dogs after type C influenza virus infection, the bronchial reactivity to acetylcholine began to increase towards day 3, reached a peak at 1 to 2 weeks, and returned to a normal level at 4 weeks [23]. In children under 2 years of age, respiratory syncytial virus is the most prevalent respiratory viral infection [24, 25, 26]. It may cause bronchiolitis with symptoms of acute airway obstruction which is often difficult to distinguish from asthma [27]. These infants go on to experience recurrent wheezing later in life [28, 29]. In adults, a variety of respiratory viruses (Rhinovirus, Parainfluenza, Influenza viruses) may trigger an asthmatic attack and enhance bronchial responsiveness [30, 31, 32]. It is generally assumed that symptoms of asthma and obstructive bronchitis in childhood following viral infections are mediated via airway inflammation and epithelial damage [33, 34, 35]. A variety of antigens, occupational stimuli and pollutants can also provoke airway inflammation [21, 36]. Mediators from mast cells, neutrophils, epithelial cells and mostly from activated macrophages can initiate and amplify the airway inflammation with increased responsiveness [10, 33, 37]. This hyper-responsiveness may last for a long time and make the bronchial surface vulnerable to antigens resulting in sustained course of obstructive symptoms after contact with antigen. More recently, a soluble factor produced by alveolar macrophages has been reported which induce histamine release from lung mast cells prolonging the asthmatic symptoms during the macrophage-mediated airway inflammation [38].

The most frequent causes of airway inflammation mentioned above provocate a non-purulent reaction characterized especially by the influx and activation of macrophages. Airway hyper-responsiveness with long-lasting course correlates with this type of inflammatory reaction. Hyper-reactivity is invariably present in patients with cystic fibrosis, chronic bronchitis and other diseases in which the airways are typically infiltrated and the process undoubtedly puru- lent [39].

Taken together, it seems, that epithelial damage and influx of neutrophils caused by for example ozone challenge may result in a short and transient airway inflammation and hyper-responsiveness by release of mediators such as leukotriens, but a macrophage-mediated inflammation may generate a self-perpetuated process with permanent bronchial hyper-responsiveness.

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