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Dry air-induced late phase responses in the canine lung periphery

A.N. Freed, N.F. Adkinson Jr

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ABSTRACT: Although controversial, late phase responses in asthmatic subjects have been reported several hours after exercise. We previously showed that exposure to dry air increases collateral system resistance (Rcs) in the canine lung periphery, and produces acute airway responses analogous to those that characterize human exercise-induced asthma. We used a dual wedged bronchoscope technique in anaesthetized male mongrel dogs to monitor Rcs in: 1) control segments continuously exposed to 200 ml·min^{-1} of 5% CO_2 in air and 2) dry air challenged segments exposed to 2000 ml·min^{-1} 5% CO_2 for 5 min. We examined Rcs at 5 min and ~5 h post-challenge in an attempt to document late phase airway obstruction. Five min after dry air challenge Rcs initially increased 114±SE 22%; contralateral control segments remained unchanged (n=9). Five hour post-challenge, Rcs in dry air exposed segments was elevated 81±20% above pre-challenge baseline ($p<0.01$); contralateral control segments did not change significantly over the 5 h period. Cell profile analyses of lavage samples at 5 hours revealed that neutrophils and eosinophils were significantly increased ($p<0.03$) in dry air challenged segments when compared to controls. Leukotriene C_4/D_4 concentration in lavage was correlated ($p<0.02$) with neutrophil infiltration. Thus, we conclude that the canine lung periphery represents a reproducible model of a dry air-induced late phase reaction.
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A number of animal models have been developed specifically to examine the mechanism(s) underlying late phase airway obstruction. Responses of sensitized guinea-pig [1, 2], rabbit [3, 4], rat [5], sheep [6], and squirrel monkey [7] to aerosolized antigen are in many respects analogous to those reported in asthmatic subjects [8, 9]. Although allergic dogs do not exhibit a whole lung late phase response [10], animals treated with a cortisol synthesis inhibitor do [11], and a delayed response has been demonstrated in the lung periphery of *Ascaris* sensitive dogs [12]. Late phase responses commonly follow the resolution of an acute response [1, 2, 5–7, 11] and begin 2–6 h after the initial exposure [2, 7, 11], although others start later [1, 5, 6]. Maximal airway obstruction usually occurs between 4–8 h post-exposure [2, 5–7], but has been reported as late as 17 h post-challenge [1]. Depending on the model, duration varies from a few hours [5, 7] to several days [1]. Similar to that seen in humans [8, 13–15], late phase reactions are sometimes more severe than acute responses [3, 5, 11], may occur in the absence of an acute response [5], and may persist longer than 12 h [1, 2, 7]. Leucocyte infiltration has been examined in several animal models, and as in humans [16–18], influx of eosinophils [1–3, 19] and neutrophils [1–4, 11, 19] are associated with late phase airway obstruction.

Bronchospasm occurs in asthmatic subjects within 10 min after a period of exercise or isocapnic hyperventilation of cold or warm dry air, and usually subsides spontaneously within 1–2 h. Although controversial [20, 21], late phase responses have been repeatedly documented and occur in some asthmatics 3–13 h after exercise [15, 21–26]. The time course of exercise-related late phase responses is similar to that reported for antigen-induced late phase reactions [15, 22, 23, 25]. Asthmatic subjects that exhibit a late phase response to allergen may [15] or may not [27] exhibit a late phase response following exercise, and exercise-associated late phase reactions may [24] or may not [15] be reproducible within subjects over time. Late phase responses can be elicited with the inhalation of distilled water aerosols as well as exercise, and hyperreactivity to methacholine challenge has been demonstrated after the late phase [15]. As with an antigen-induced late phase reaction [8, 15], an exercise-associated late phase response may occur in the absence of an acute response [21, 26]. Unlike the late phase response to antigen, an exercise-associated late phase response is usually less severe than

the immediate response [15, 22–24], although both are believed to primarily involve small airways [13, 22]. Although a cellular basis for exercise-associated late phase response has not been demonstrated, neutrophil chemotactic activity has been detected in the plasma of asthmatic subjects during the late phase [25].

We previously showed that exposure to dry air increases collateral system resistance (Rcs) in the canine lung periphery, and produces acute responses analogous to those that characterize human exercise-induced asthma [28, 29]. We now present data indicating that a "late phase" response is detectable in the canine lung periphery 5 h after dry air challenge. This late response is characterized by an elevation in Rcs, neutrophil and eosinophil infiltration, and increased concentrations of leukotriene C_4/D_4 (LTC_4/D_4) in dry air exposed segments.

Materials and methods

Dogs were handled in accordance with the standards established by the USA Animal Welfare Acts set forth in DHEW (NIH) guidelines and the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health's Animal Care and Use Committee. Male mongrel dogs (mean weight±SE = 19.7±0.58 kg, n=17) were anaesthetized with pentobarbital sodium (30 mg·kg^{-1}). Supplemental anaesthesia was administered intravenously as required. Dogs were intubated with a dual portal endotracheal tube and ventilated with a Harvard constant volume respirator. End-expiratory CO_2 was monitored with a CO_2 analyser (Beckman LB-2) and maintained around 4.5% by adjusting the respiratory frequency. A telethermometer (YSI 43) was used to monitor body temperature (T_b) *via* a rectal probe.

Measurements of collateral system resistance

Two fibreoptic bronchoscopes (Olympus BFA-4B2, 5.5 mm OD) were inserted through airtight portals of the endotracheal tube, and visually guided into sublobar segments of both lungs until each tip obstructed a bronchus. Ventilation of these segments could then occur only through collateral channels [30]. A 5F double-lumen catheter was threaded through the suction port of each bronchoscope and used to measure pressure (P_b) at the tip of the bronchoscope. Room temperature (22–23°C) dry air with 5% CO_2 was delivered at a constant flow of 200 ml·min^{-1} through the port, around the catheter, and into the wedged sublobar segment. Rcs under quasi-static conditions was calculated from measurements made at functional residual capacity (FRC) [30]. With the ventilator stopped, P_b plateaus at a pressure above the alveolar pressure (P_a) in the surrounding unobstructed lung. At this time, $(P_b-P_a)/200$ ml·min^{-1} = the resistance through the collateral system (Rcs). $P_a=0$ when measurements are made with the ventilator stopped at FRC.

Time course of the acute airway response to dry air

After establishing a stable baseline Rcs, wedged sublobar segments in 8 dogs (9 lungs) were exposed for 5 min to 2000 ml·min^{-1} of 5% CO_2 in air. Airflow was then returned to 200 ml·min^{-1}. Rcs was recorded at 2 and 5 min post-challenge, and was monitored every 5 min for 1 h after the dry air exposure.

Acute and late airway responses to dry air

After a stable baseline Rcs was established in wedged sublobar segments of 9 dogs, dry air challenge was randomly performed in one of the two selected segments by increasing collateral airflow from 200 ml·min^{-1} to 2000 ml·min^{-1} for a 5 min period of exposure. Airflow was then returned to 200 ml·min^{-1}. Rcs was monitored for 15 min, and both bronchoscopes were removed from the animal. In the control segment, airflow remained at 200 ml·min^{-1} throughout the experiment. Approximately 4.5 h later, with the use of an airway map made during the initial wedging procedure, the bronchoscopes were rewedged in the same sublobar segment and Rcs was recorded until a stable baseline was established. At that time, approximately 5 h post-challenge, both sublobar segments were lavaged. Lavage fluid was centrifuged, the cell pellet resuspended for differential cell analysis, and the supernatant used for mediator assays as previously described [31, 32]. The immunoreactive material was previously characterized using high performance liquid chromatography [31, 33].

Bronchoalveolar lavage and differential cell counts

Lavage was performed 5 h post-challenge using two 40 ml and one 20 ml aliquots of warm (37°C) modified Hanks' balanced salt solution (Ca^{++} and Mg^{++} free). Fluid was delivered *via* the suction port of the bronchoscope and was gently suctioned from the wedged segment using a 20 ml syringe. Average return of fluid was 46.3±4.3 ml (n=18). Lavage samples were stored at 4°C until the conclusion of the experiment, and centrifuged at 4°C for 10 min at 1300 rpm. The cell pellet from a 5 ml sample was resuspended in 1 ml of supernatant and a 10 µl sample was placed on a haemocytometer to determine total cell number. A cytospin was used to prepare slides from this concentrated cell sample, and blinded differential cell counts of macrophages, lymphocytes, neutrophils, eosinophils, and epithelial cells were performed after staining with Diff-Quik. The bulk of the supernatant was saved for mediator assays.

Mediator concentrations in lavage fluid

Determinations of prostaglandin D_2 (PGD_2), thromboxane B_2 (TXB_2), and leukotrienes C_4/D_4 (LTC_4/D_4) were carried out as follows: lavage fluid sample was

concentrated using a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA) and eluted in 4 ml of methanol. This sample was centrifuged and the supernatant evaporated to dryness. The dried sample was then reconstituted to 1.0 ml with 0.1% gelatin-PBS. The mediator was then measured using a competitive radioimmunoassay system as previously described [31–34].

Statistical analyses

Rcs data were analysed using repeated measures analysis of variance (ANOVA) techniques and Duncan's multiple range test. Because of the dilution effects associated with lavage experiments, differential cell count and mediator concentration data may not meet the assumptions necessary for parametric analysis. Thus, the Wilcoxon sign-rank test was used to compare control and experimental mediator concentrations and cell counts in lavage samples. Spearman's rank analysis was used to test for correlations between changes in Rcs and mediator release and changes in differential cell numbers and mediator concentrations in control and experimental lavage samples. Statistical significance was judged at $p<0.05$ in all cases.

Results

Time course of the acute airway response to dry air

Rcs increased from 0.58±0.07 $cmH_2O \cdot ml^{-1} \cdot s$ to 1.64±0.23 $cmH_2O \cdot ml^{-1} \cdot s$, an average increase of 199±42% (n=9) 5 min post dry air challenge. Rcs slowly returned toward baseline within the 60 min post-challenge period, and was only 46±13% above the original baseline at the end of that 1 h period (fig. 1).

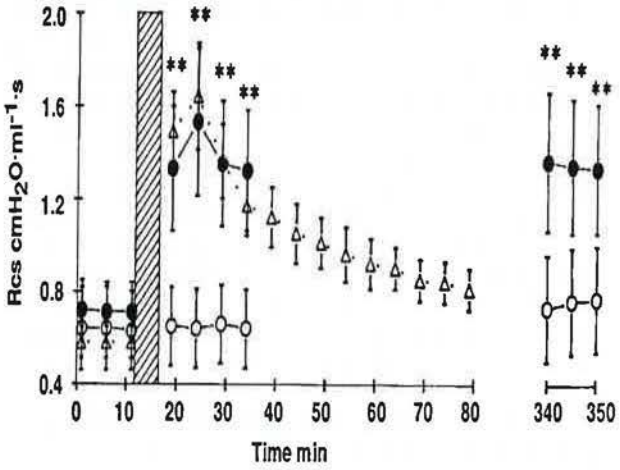

Fig. 1. – Acute and late changes in collateral system resistance (Rcs) over a 350 min period in control and dry air challenged contralateral sublobar segments in which bronchoscopes were removed and then replaced 5 h later (n=9). Dry air responses in a second control group were monitored for 60 min post-challenge (n=9). Vertical bar = 2000 $ml \cdot min^{-1}$ challenge. **: $p<0.01$ statistically different from control values. o—o : Control; ●———● : Flow; Δ . . . Δ : Flow-60.

Acute and late airway responses to dry air

ANOVA indicated that a significant interaction ($p<0.0001$) existed between time and treatment effects. Rcs rose from 0.71±0.13 to1.53±0.32 $cmH_2O \cdot ml^{-1} \cdot s$ ($p<0.01$, n=9), which represents an average increase above baseline of 114±22% (n=9) 5 min after challenge (fig. 1). Rcs in the contralateral control segment did not significantly change during that time period. Five hours post-challenge, baseline Rcs of the dry air exposed segment was significantly elevated ($p<0.01$) above the original baseline. Four animals increased 110–150% above the original baseline, three increased between 50–80% above baseline, and one animal fell ~30% below baseline. In contrast, Rcs of the contralateral control segment 5 h post challenge was not significantly different from its original baseline value.

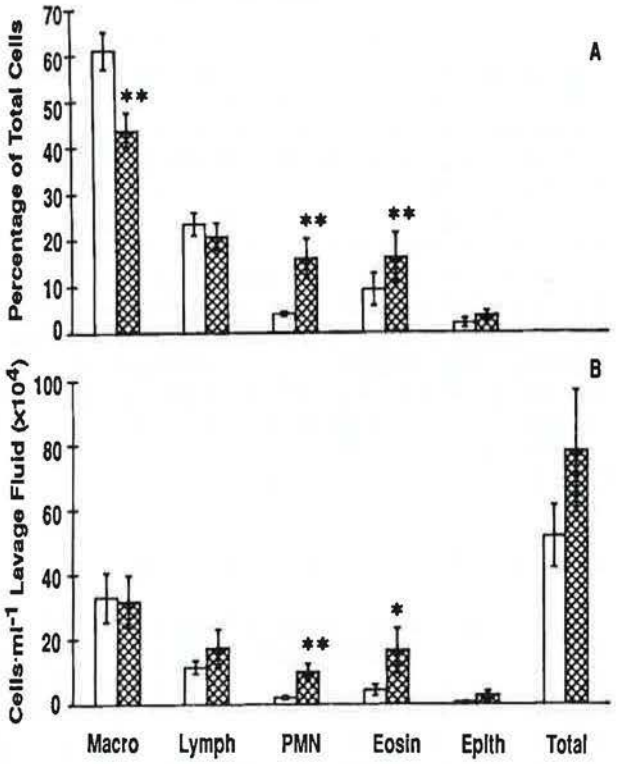

Fig. 2. – A) Differential cell counts expressed as percentage of total cells recovered in lavage fluid from control and dry air exposed sublobar segments 5 h post-challenge. B) Differential cell counts from (A) expressed as $cells \cdot ml^{-1}$ of lavage fluid. Macro: macrophage; Lymph: lymphocyte; PMN: neutrophil; Eosin: eosinophil; Epith: epithelial cell. n=9. *:p=0.05; **:p=0.01. □ : Control; ⊠ : Flow.

Bronchoalveolar lavage and differential cell counts

Differential cell counts are expressed as percentage of total cells (fig. 2a) and as $cells \cdot ml^{-1}$ of lavage fluid (fig. 2b) recovered from control and dry air exposed sublobar segments 5 h post-challenge. On a percentage basis, macrophages decreased (p=0.004), and neutrophils (p=0.005) and eosinophils (p=0.014) increased

significantly in lavages taken from dry air exposed segments when compared to their contralateral control segments. Significant changes were detected only in neutrophils (p=0.005) and eosinophils (p=0.033) when the data were analysed as cells·ml^{-1} of lavage fluid recovered.

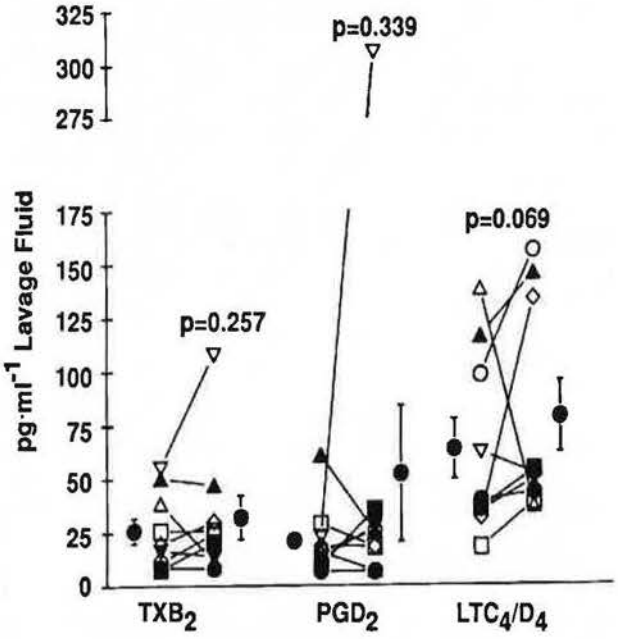

Fig. 3. – Concentrations of thromboxane B_2 (TXB_2), prostaglandin D_2 (PGD_2), and leukotriene C_4/D_4 (LTC_4/D_4) in lavage fluid recovered from control (left) and dry air exposed sublobar segments (right) 5 h post-challenge. Individual values with mean±SE for each mediator, n=9.

Mediator concentrations in lavage fluid

Concentrations of PGD_2 (p=0.339) and TXB_2 (p=0.257) in lavage samples taken 5 h post dry air challenge were not significantly elevated above that detected in control samples (fig. 3). Although not statistically significant, concentrations of LTC_4/D_4 (p=0.069) tended to be greater in segments exposed to dry air challenge.

Correlation between acute airway response and late phase mediator release

Concentrations of PGD_2 and TXB_4 recovered in lavage fluid 5 h post-challenge were not significantly associated with the percentage change in Rcs recorded 5 min post-challenge. However, a correlation (r=0.67, p=0.024, n=9) was detected between concentrations of LTC_4/D_4 recovered in lavage fluid 5 h post-challenge and acute responses recorded 5 min post-challenge (fig. 4).

Correlation between late airway response and late phase mediator release

Concentrations of PGD_2, TXB_4, and LTC_4/D_4 recovered in lavage fluid 5 h post-challenge were not significantly correlated with the percentage change in Rcs recorded 5 h post-challenge.

Correlation between late phase cell influx and mediator release

Differences (dry air-control) in PGD_2 and TXB_4 concentrations were not associated with differences in eosinophil and neutrophil numbers in lavage fluid recovered 5 h post-challenge. Although LTC_4/D_4 concentrations were not correlated with eosinophils, they were significantly correlated (r=0.70, p=0.017, n=9) with differences in neutrophil number in lavage fluid recovered 5 h post-challenge (fig. 5).

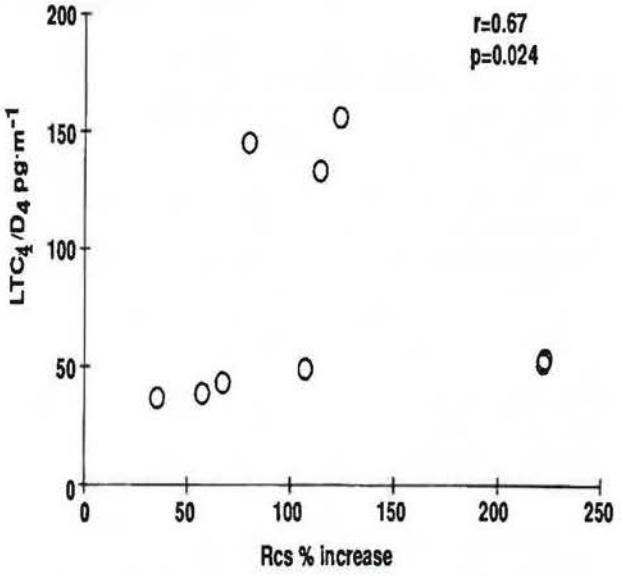

Fig. 4. – Concentration of leukotriene C_4/D_4 (LTC_4/D_4) in lavage fluid recovered 5 h post-challenge *versus* the percentage change in Rcs recorded 5 min post-challenge.

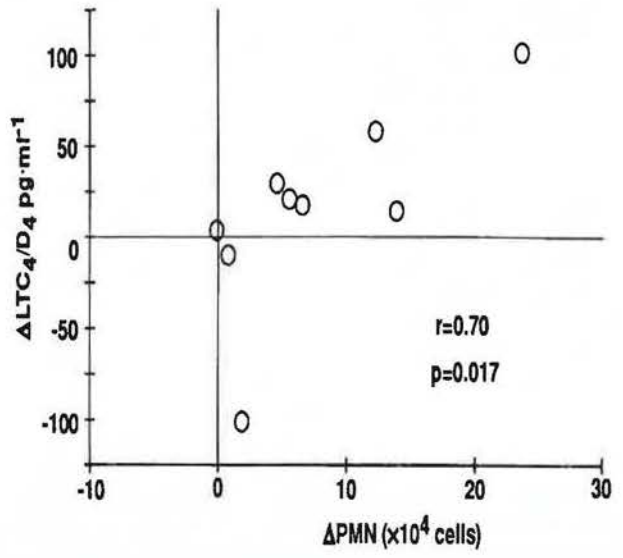

Fig. 5. – Difference (dry air-control) in leukotriene C_4/D_4 (LTC_4/D_4) concentrations *versus* the difference in neutrophil (PMN) numbers in lavage fluid recovered 5 h post-challenge.

Discussion

Late phase airway obstruction analogous to that reported in human subjects 3–13 h after exercise [15, 21–26] occurs in the lung periphery of dogs. Resistance to airflow in the lung periphery is significantly elevated 5 h post dry air challenge when compared to unexposed control sublobar segments (fig. 1.). Five hours after the initial exposure, the average Rcs of dry air exposed segments was elevated 81±20% above the original baseline. In contrast, Rcs of contralateral control segments 5 h post-challenge was 24±19% above its original baseline value. This shift in baseline control values was largely due to one animal. Without that animal, control baseline increased an insignificant 6.5±8% (n=8) over the 5 h period separating challenge from lavage.

A key component of the late asthmatic response to antigen is the cellular infiltration that is associated with the deteriorating pulmonary function that occurs 3–5 h after challenge. Airway infiltration of eosinophils and neutrophils [16–18] are believed to play an important role in the development of this late phase response. We demonstrated that a late phase cellular infiltration characteristically accompanies the delayed obstructive response to dry air challenge in the canine lung periphery (fig. 2). Because bronchoscopy itself may produce inflammation and trigger neutrophil influx [35], bronchoscopes were removed during the 5 h period separating dry air challenge from lavage. As evident in figure 2, control cell profiles were markedly different from those obtained from segments exposed to dry air, and were similar to control profiles obtained from experiments focusing on the acute airway response [31, 32]. Similar to human [16–18] and animal [1–4, 11, 19] model late phase responses to antigen, both neutrophil and eosinophil numbers were significantly elevated 5 h after dry air challenge. Similar cellular events have not yet been reported in human subjects exhibiting exercise-associated late phase responses.

Although a dearth of data quantitating pulmonary mediator metabolism exits, mediator release *via* acute phase-associated inflammation is believed to play an important role in producing late asthmatic responses to allergen [36]. Experiments involving a variety of pharmacologic interventions suggest that leukotrienes play a major role in the development of a late phase response in allergic sheep [6, 37]. Although no late phase changes in pulmonary mediator activity have been reported after exercise, changes in plasma neutrophil chemotactic activity have been demonstrated [25]. In our study we found that of the three mediators examined, LTC_4/D_4 tended to be elevated 5 h post-challenge when compared to control (fig. 3). Although it is unlikely that the bronchoscope comes into contact with airways that participate in a late phase response, we cannot rule out the possibility that bronchoscopy caused mediator release only from inflammed dry air exposed airways and not from control airways. The magnitude of the response in our canine model was significantly correlated with LTC_4/D_4 concentrations 5 h post-challenge. This suggests that the acute response may affect late phase mediator release *via* its role in cellular infiltration, and the degree of eosinophil and neutrophil influx may reflect the magnitude of acute mediator production. In addition, changes in neutrophil number were significantly associated with late phase changes in LTC_4/D_4 concentrations. However, increases in neutrophils and LTC_4/D_4 may be independent indicators of the severity of airway injury, and thus unrelated causally. Although we know of no data concerning mediator metabolism of canine neutrophils and eosinophils, human eosinophils and neutrophils are capable of producing these leukotrienes [38]. However, LTC_4 is also a major arachidonic acid metabolite produced by canine epithelial cells [39], and the significant correlation between LTC_4/D_4 and neutrophils observed in this study may reflect neutrophil-epithelial cell interactions.

The existence of exercise or dry air hyperventilation associated late phase responses is no longer controversial. However, because of a number of inconsistencies that exist, the relationship between exercise and antigen-induced late reactions is controversial [21]. First, some asthmatics exhibit late responses to antigen but not exercise [27]. This is not surprising considering the heterogeneity within the group of individuals we typically classify as "asthmatic". It is important to emphasize that within the group a subset of individuals exist that respond to both stimuli [15]. Second, it has been claimed that unlike antigen, exercise does not elevate airway reactivity. However, this is true in respect to acute responses only, because increased reactivity to methacholine challenge has been demonstrated after exercise and distilled water-associated late phase reactions [15]. Finally, exercise associated late reactions are said to be nonspecific phenomena that are independent of an initial response and are unrelated to physical exertion. This is not unexpected because "exercise-induced" acute responses, as illustrated by hyperventilation [40], appear unrelated to physical exertion. In addition, antigen associated late phase reactions occasionally do occur in the absence of an acute response [8, 13–15]. However, we would not conclude from this observation that specific late phase reactions are independent of acute events. These examples do suggest that any stimulus that initiates mediato release, with or without an immediate response, may produce a late reaction. In light of our results in the canine lung periphery, *in vivo* assays focusing on late phase-associated mediator release in human lungs are needed to adequately evaluate this question.

In summary, local insufflation of dry air through the peripheral lung of anaesthetized dogs results in late phase responses similar to those experienced by asthmatic subjects after exercising or after hyperventilating cold dry air. In this animal model, the time course of response is similar to that in man, eosinophil and neutrophil infiltration occurs concomitantly, and mediator release is associated with this late phasereaction. We conclude that the canine lung periphery represents a unique model of a dry air-induced late phase response, and may provide insight concerning

the underlying mechanisms of both acute and late phase airway obstruction.

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Réponses tardives induites par un courant aérien sec à la périphérie du poumon de chien. A. Freed, N. Adkinson.

RÉSUMÉ: Quoique contestées, des réponses tardives ont été rapportées plusieurs heures après l'effort chez les sujets asthmatiques. Nous avons démontré antérieurement que l'exposition à l'air sec augmente la résistance du système collatéral (Rcs) à la périphérie du poumon de chien, et produit des réponses aiguës des voies aériennes analogues à celles qui caractérisent l'asthme induit par l'effort chez l'homme. Nous avons utilisé une technique faisant appel à un bronchoscope à double blocage, chez des chiens mâles batards anesthésiés, pour suivre Rcs: 1) dans des segments de contrôle exposés de façon continue à 200 $ml \cdot min^{-1}$ de 5% de CO_2 dans l'air, et 2) dans des segments soumis à l'air sec exposés à 2000 $ml \cdot min^{-1}$ de 5% de CO_2 pendant 5 minutes. Nous avond examiné Rcs à 5 min et ±5 h après la provocation, pour tenter de documenter l'obstruction des voies aériennes en phase tardive. Cinq minutes après une provocation à l'air sec, Rcs augmente initialement de 114±SE 22%; les segments contrôle contro-latéraux restent inchangés (n=9). Cinq heures après la provocation, Rcs, dans les segments exposés à l'air sec, est élevé de 81±20% au-dessus de la valeur basale préalable à la provocation ($p<0.01$); les segments contrôle contro-latéraux n'ont pas changé significativement pendant la période de 5 heures. Les analyses des profils cellulaires des échantilions de lavage prélevés après 5 h révèlent une augmentation significative des neutrophiles et des éosinophiles ($p<0.03$) dans les segments soumis à une provocation par l'air sec, par comparaison aux contrôles. La concentration de leukotriène C_4/D_4 dans le lavage est en corrélation avec l'infiltration neutrophilique ($p<0.02$). Donc, nous concluons que la périphérie du poumon de chien représente un modèle reproductible pour la réaction en phase tardive après provocation par l'air sec.
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