Effect of the sensitization to 
*Derma*tophagoïdes pteronyssinus* on the inflammatory response and bronchoconstrictive of brown Norway rats

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**Abstract** — Asthma is a chronic inflammatory disease whose clinical consequences are airway obstruction. We sensitized rats brown Norway with allergenic proteins from *Dermatophagoïdes pteronyssinus* (Der P1 and Der P2) for a better understanding of the pathophysiological mechanisms of this disease. Male brown Norway rats (300-400 g) were sensitized by 2 subcutaneous injections with proteins allergens (Der p1 and Der p2) (50 IR/ml) (Stallergenes AS, France) and Al2O3 at days 0 (D0) and 3 (D3), followed at D17 by intratracheal instillation of Der p. Control (C) rats (n=5) underwent the same protocol but with saline solution instead of Der p. Witness (W) rats (n=5) were not submitted to any treatment. At D24, enhanced expiratory pause (Penh), used as an index of airway resistance, was measured using a barometric plethysmograph for conscious animals. At D25, rats were killed, a bronchoalveolar lavage (BAL) was performed, and isometric contraction was measured on rings isolated from trachea (T), extrapulmonary (EPB) and intrapulmonary bronchi (IPB) using an organ bath system. Maximal contraction (Fmax) and LogEC50 were derived from cumulative concentration response curves to -8 to -3 LogM carbachol (CCh). The parameters measured are: airways resistance, inflammatory cells infiltration in BAL, allergen-specific response, and hyperresponsiveness and hypersensitivity of the airways to muscarinic agonists.

In BAL fluid, cellular density was significantly higher in sensitized rats (334 ± 52 cells/µl) versus Control (217 ± 18 cells/µl) and versus Witness (165 ± 52 cells/µl) rats, as was the percentage of eosinophils (S: 8.75 %; C: 1.74 %; W: 0.7 %) and mast cells (S: 0.5 %; C: 0.2 %; W: 0 %). Allergen challenge increased Penh in sensitized rats, but not in Control and Witness rats. In vitro stimulation by Der p induced contraction of trachea, EPB and IPB rings isolated from S, but not C and W rats. In response to metacholine (MCh) challenge, MCh concentration (in LogM) inducing 300 % increase in Penh was significantly lower in sensitized rats (-4.36 ± 0.44) and Control (-3.92 ± 0.54) versus Witness rats (-2.86 ± 0.55). LogEC50 is significantly lower in sensitized rats (-6.22 ± 0.03; versus Control (-5.64 ± 0.02); and Witness rats (-5.23 ± 0.02). Sensitized rats showed (i) in vivo and ex vivo specific bronchoconstriction to allergen stimulation, (ii) in vivo hyperresponsiveness and ex vivo hyperreactivity and hypersensitivity to cholinergic stimulation, and (iii) increased proportion of eosinophils and mast cells in BAL fluid, indicating that such sensitized rats are a relevant model of asthma.

**Keywords** — asthma, *Dermatophagoïdes pteronyssinus*, airways.

**I. INTRODUCTION**

Asthma is a chronic inflammatory airway disease which is accompanied by a hyperresponsiveness of airway smooth muscle. Using animal model was to understand the pathophysiology of this disease but each animal model studied has advantages and disadvantages when compared to human asthma. It is the same for the type of allergen and the exposure mode that also are factors that could cause differences. Bronchoconstriction, eosinophilic infiltration, increased IgE and mucus production are the four basic elements of human asthma, the animal model must reflect if possible least 3 of these 4 components [1]. In order to understand the pathophysiological mechanisms of this illness, we used an animal type, the brown Norway rat stimulated by intra tracheal instillation with the major proteins of *Dermatophagoïdes pteronyssinus*, an allergen responsible for the human sensitization when inhaled.

**II. ANIMALS AND METHODS**

**Animals**
The sensitized rats, control and witness are brown Norway rats male that weigh between 300 and 400g. For each experimental condition the number of rats varies from 10 to 15.

**Active sensitization to Der p in vivo**
The brown Norway rats were actively sensitized by subcutaneous injection of 0.5 ml of major allergen extract (Der p1 and Der p2) of 10 µg/ml in a solution
of aluminum hydroxide (Al₂O₃) of 13 mg/ml, at day 0 and day 3. At the 17th day, they received a stimulation by intratracheal instillation of 250 μl of Der p to 10 μg/ml in physiological solution.

The control rats are treated in the same conditions by subcutaneous injection with 0.5 ml of Al₂O₃ only at day 0 and day 3, followed by an intratracheal instillation at D17 of 250 μl of physiological solution while witness rats don’t receive any particular treatment.

Provocation tests of the brown Norway rats to agonists

One week after the final sensitization (D24), the sensitized animals and non sensitized are placed in a barometric plethysmograph for vigil animal (BUXCO, Troy, NY). A 10 min period of adaptation in the chamber is observed, then the animal receives for 5 min physiological solution aerosol spray, followed by 10 min of recording. The rats receive again increased concentrations of Der p or metacholine aerosol spray (MCh) for 5 min followed by 10 min recording. At the stoppage of each stimulation the Penh is continually recorded and a point is made every 10 seconds. This protocol of experience has been applied to every individual of each group. The Penh measures the airways’ resistance during the phase called «expiratory pause ».

Bronchoalveolar lavage technique

A week after the intratracheal stimulation (D25) the rats are anaesthetized by intraperitoneal injection of a mixture of Rompun® 2% (4mg/Kg) and of Imalgen® 5% (60mg/Kg). The trachea is cannulated then the lungs are washed 5 times with 4 ml of sterile physiological solution. The cellular density of the collected bronchoalveolar washing liquid is valued on a cell of Mallassez. The cytological formula is achieved by the numbering of cells on slides colored by Diff quick after 5 minutes of cytocentrifugation to 450 rpm, identical protocol to the one used for the analysis of bronchoalveolar lavage (BAL) of human patients.

Obtaining tracheal, extra and intrapulmonary bronchi rings

brown Norway male Rats of 300 to 400g are sacrificed by cervical dislocation, then the heart - lung block is immediately put in a solution of Krebs-Henseleit (KH) of 2 mM of Ca²⁺. Trachea, extra intrapulmonary (EPB) and intrapulmonary bronchi (IPB) rings measuring 3 mm long are gotten after dissection under binocular magnifying glass. For each experience one gets 2 rings of trachea, 2 rings of left and right extra pulmonary bronchi and 2 rings of left intrapulmonary bronchus. Animals were treated and sacrificed according to national guidelines, with approval of the local ethical committee.

Isometric contraction measurement

Rings are placed in bath for isolated organs containing a KH solution in the presence of carbogen. They are connected to a force transducer of isometric contraction (IOS EMKA France technology). Each ring is stretched by an initial maximal load of 2.5 g for the trachea and 1.5g for the bronchi for 1 h. After this equilibration rings are stimulated with the acéthylcholine (ACh) 10⁻⁶M for 1 h in order to get a maximal contraction serving to standardize the responses in the different experimental conditions as previously described [2]. Rings were then washed with fresh KH solution to eliminate the ACh response. After the tension returned to baseline, Rings of trachea, EPB and IPB of actively sensitized rats to Der p and non sensitized (control and witness) are stimulated by a fixed concentration (0.05 μg/ml) of antigens Der p1 and Der p2 of Dermatophagoides pteronyssinus. The response to Der p is expressed relative to the dry weight of the ring.

Ethical Considerations

The experiment Protocols on rats have been lead according to recommendations of the national guide of animal experimentation, with the approval of the ethics committee for the treatment and the sacrifice of animals.

Data analysis and statistics

Data are given as mean ± SEM. The maximal contraction Fmax was taken as the apparent maximal response, i.e., the response obtained with the maximal concentration used, even though the CRC had not reached a plateau. Overall differences in CRC were performed by ANOVA test. Fmax is compared using Student's t tests for quantitative variables and χ² tests for qualitative variables. Results were considered significant at P < 0.05

III. RESULTS

Inflammatory cells Distribution in the bronchoalveolar lavage

The cellular density of the bronchoalveolar lavage liquid (BAL) is rated on a cell of Mallassez. Results gotten in each experimental condition are homogeneous. This cellular density was more important in the sensitized rats compared to the control rats and witness. The analysis by category of cells showed that the number of eosinophil counts and lymphocytes was also more elevated in the sensitized rats. The stimulation to Der p and the hydroxide of aluminum shows mast cells in the BAL. The global comparisons by ANOVA for repeated measures indicate that the gotten values are statistically different. Table1
Table I: Distribution of the different categories of cells in the brown Norway rats LBA

<table>
<thead>
<tr>
<th></th>
<th>Sensitized rats (n=15)</th>
<th>Control rats (n=12)</th>
<th>Witness rats (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Cells/µl</td>
<td>334000*</td>
<td>217000£</td>
<td>165000</td>
</tr>
<tr>
<td>Macrophages</td>
<td>243820 (73%)*</td>
<td>169650 (78%)£</td>
<td>157575 (95%)£</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>55945 (17%)*</td>
<td>38606 (18%)£</td>
<td>5362 (3.3%)£</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3340 (1%)</td>
<td>4893 (2%)</td>
<td>825 (0.5%)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>29225 (8.7%)*</td>
<td>3808 (1.7%)£</td>
<td>1237 (0.7%)£</td>
</tr>
<tr>
<td>Mast cells</td>
<td>1670 (0.5%)*</td>
<td>544 (0.2%)£</td>
<td>0</td>
</tr>
</tbody>
</table>

(*) P <0.05 sensitivities versus witness, (£ )P <0.05 controls versus witness

Effect of the active sensitization on the response to Der p in vivo

The base Penh gotten after the nebulization of the physiological solution is the same in the 3 groups of rats. With the control and witness rats the stimulation to Der p does not entail any response, whereas with the sensitized rats it induces a specific response, dependent concentration. (fig1)

Figure 1: Specific response of the brown Norway rats to Der p in vivo.

The sensitized Norway brown rats and non sensitized to Der p are stimulated by inhalation of solution of increasing concentration Der p (10^-4 to 10^-7M) for 5 min. Airway resistance is expressed in percentage of the Penh gotten for the physiological solution. (■) Rats treated with Der p + Al₂O₃, n = 15; (●) rats treated to Al₂O₃, n=12; (▲) untreated rats, n=10. The gotten values represent the average ± SEM in each experimental condition. (+) P <0, 05 versus control. (‡) P <0, 05 versus witness.

Effect of the active sensitization on the response to the Der p in vitro

Der p induces in vitro, a contractile, transient and specific response on the airways of sensitized rats (fig 2A). The amplitude of this contraction depends on the position of the tissue on the bronchial tree. It is more important on the IPB than on the trachea and the EPB (fig 2B). The relaxation time (TR10) that corresponds to the time for which the contraction loses 90% of its maximal value is weaker on the IPB (8, 96 ± S 1, 02 min) and the EPB (10, 41 ±S 3, 53 min), than on the trachea (17, 3 ±2, 85 min)

A: Typical response graph

B: The contractile response to Der p

Figure 2: The specific response to Der p in vivo

A: the response Typical graph to Der p (0,05 µg/ml) of trachea rings. B: The contractile response to Der p is normalized in relation to the dry weight of the ring, represents the average ± SEM (n=15). The bars of error represent the SEM. The responses of rats control’s rings (n =12) and witness (n =10) are not different from zero (non shown data).

Non specific response to the metacholine in vivo of rats actively sensitized

The base Penh gotten with the physiological solution is similar in the 3 groups of rats. The dose – response curves to the metacholine (MCh) show an increase concentration-dependent of airway resistances in the 3 groups of rats, but this increase is higher in the rats treated with Der p in relation to control rats which in turn have a more elevated response than witness rats.
For an increase of the Penh to 300%, methodology previously used by Hamelmann E, et al [3], the necessary MCh concentrations that we got in our survey are respectively of $10^{-5}$M, $10^{-4}$M and $10^{-3}$M, in sensitized rats, control and witness, which corresponds to a displacement of the curve toward the left (figure 3).

![Figure 3: The response of the brown Norway rats to the metacholine in vivo.](image)

Table II: EC50 of the stimulation to the Carbachol (CCh) on airway rings of brown Norway sensitized rats, control and witness.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sensitized Rats</th>
<th>Control Rats</th>
<th>Witness Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 ± SEM</td>
<td>-6.43 ± 0.03*#</td>
<td>-6.35 ± 0.04#</td>
<td>-6.10 ± 0.05 # -6.22 ± 0.03*#</td>
</tr>
<tr>
<td>Left EPB</td>
<td>5,33 ± 0.02</td>
<td>5,64 ± 0.02</td>
<td>-5.26 ± 0.05</td>
</tr>
<tr>
<td>Right EPB</td>
<td>5,34 ± 0.02</td>
<td>5,33 ± 0.03</td>
<td>-5.26 ± 0.05</td>
</tr>
<tr>
<td>IPB</td>
<td>5,34 ± 0.02</td>
<td>5,33 ± 0.03</td>
<td>-5.26 ± 0.05</td>
</tr>
</tbody>
</table>

* p <0.05 (sensitized vs control); # p <0.05 (control vs witness); $ p <0.05 (control vs witness)

Rings of trachea, EPB and IPB of brown Norway rats are stimulated with the cumulative concentrations of carbachol. The gotten contractile responses are normalized in relation to the contractile response induced by the maximal concentration of CCh ($10^{-3}$M). The EC50 represents the concentration of CCh which entails 50% of maximal response. Sensitized rats (n =15), control rats (n=10) and witness rats (n=10).

IV. DISCUSSION

The inflammatory reaction induced by the active sensitization with Der p

In our model the active sensitization with Der p+Al$_2$O$_3$ or with the Al$_2$O$_3$ alone led to an important inflammatory cellular reaction. With the brown Norway sensitized rats with Der p by intratracheal instillation the cellular density is 1.53 times more elevated than with control rats and 2.02 times more elevated than with witness rats.

The cellular analysis showed homogeneity of the cellular density for each group of rats. The cellular density is globally more elevated in sensitized rats with Der p compared to the other groups. The analysis of the different categories of cells shows a meaningful increase of macrophages, of lymphocytes, of eosinophil counts and especially of mast cells in the sensitized rats compared to witness rats. However the comparison between the sensitized rats and control rats doesn't show a meaningful difference. The increase of macrophages and lymphocytes would therefore be due to a non specific effect of the Al$_2$O$_3$.

The rate of eosinophil counts shifted from 1,07 to 3,07 and 23,28 to 3808/ul in control rats and to 29225/ul in rats sensitized with Der p. This rate is therefore respectively 3, 07 and 23,62 times more elevated in control rats and sensitized compared to witness rats.

Many surveys showed a meaningful increase of eosinophil counts in the BAL of sensitized animals [4; 5]; with large fluctuations with regard to the rate and the kinetics of emergence. Indeed on mice sensitized by Drematophagoides farinea (Der f), Yu et al [5] observed that eosinophil counts represented 43% of cells of the BAL. The number of eosinophil increased...
progressively to reach this rate 72 hours after the stoppage of the stimulation then it slowly declined. Some similar results have been observed by Bischof et al. [6] on sheep sensitized with the total extracts of mites in intra nasal instillation. Thus they indicated the eosinophil represented between 10 and 33% of all the cells of the BAL in the sensitized sheep against only 0 and 3% in control sheep. In our experiment we found that the eosinophil represented 8, 75% of cells of the BAL after only 3 weeks of sensitization with 2 subcutaneous injections followed by an intra tracheal instillation. It represents an increase of 23, 62% compared to witness rats. The differences between these results could be bound to the type of allergens used (Ovalbumin, Der f, or Der p1 and 2), to the associate adjuvant (Al3O3 or vaccine of B pertussis), to the way of administration and the duration of the immunization. Thus the sensitization entails an infiltration of eosinophil counts in the BAL [7] their rate depends on the way of sensitization. It can thus shift from 47 respectively to 62% for a systemic way and intra tracheal one according to Singh et al. [8].

In our experiment mast cells, lymphocytes and eosinophil counts are all increased in the BAL of sensitized animals. Little research brought back a simultaneous increase of different categories of inflammatory cell in particular of mast cells in the BAL. On actively sensitized sheep with extracts of Der p, Snibson et. al., [9] brought back an increase of mast cells in the airways partition and in the alveolar septum in addition of the remodeling of the smooth muscle of the airways. Similarly on mouse models sensitized to cat allergens Grundström J et al reported an airway hyperreactivity, an infiltration of neutrophils, eosinophils, and lymphocytes in the bronchoalvelar lavage, all coupled with an increase cytokines IL17, IL5, IL4 and tissue remodeling [10]. On the other hand the research of Walls et al., [4] didn't show an increase of the mast cells on guinea pigs sensitized with the ovalbumin. They didn't likewise show an increase of macrophage, lymphocytes or epithelial cells in the BAL. These results sometimes contradictory could be bound to the animal species, to the type of exposure and the excitant used.

It has been proved that the increase of eosinophil counts is correlated to the bronchial hyperreactivity and the increase of the cytokines rate in the peripheral blood and in the BAL. These cytokines (IL4, IL5 and IL13), synthesized by the Th2 lymphocytes are responsible for the induction of the synthesis of immunoglobulin myeloma, of the recruitment and the activation of eosinophil [11 ; 12].

**The specific response induced by the sensitization to Der p**

The inhalation of the allergen to the vigil animal entailed an increase of the airways resistance in the sensitized rats whereas in control and witness rats it didn't have any effect. This response is therefore specific, dependent-concentration in the vigil animal. In vitro, the application of Der p on rings of trachea, EPB and IPB of sensitized rats also gave a specific contractile response, variable along the bronchial tree, since it is more important on the IPB that on the trachea and the EPB.

In order to clarify the implied mechanisms in the pathology of the asthma, several research on the asthmatic animal types have been realized. Inter species' differences: the multiplicity of the immunization ways, the type of allergens, as well as concentrations administered sometimes lead to the divergent results and even contradictory. In the sensitized sheep by subcutaneous way with Der p, Bischof et al [6] only observed 50 to 60% of specific response whereas in our survey 100% of the sensitized rats reacted to the stimulation to Der p. The induced response to Der p resulted in vivo in an increase of the Penh, reflecting the increase of airways resistances. Our results were in agreement with those of Singh et. al. [8] that showed an increase of the Penh specifically on the sensitized animals. Similar results have been found on different types of animals. On guinea pigs sensitized by intraperitoneal injection of crude Der p extracts, Hsiue et al., [7], got an anaphylactic bronchoconstriction in response to the intra venous injection of Der p extract.

**The induced non specific response by the sensitization to Der p**

On the vigil animal the métacholine (MCh) has also induced a dependent-concentration response that was characterized by an increase of the Penh. The amplitude of this response varied according to the experimental conditions. So for a given concentration of MCh we noticed a hyperreactivity in the sensitized rats compared to control and witness rats. While comparing the effect of the Al3O3, we noticed that the Penh was also significantly higher in control rats than in witness rats. The role of the Al3O3 was to stimulate in a non specific manner the immune system. Besides the hyperreactivity, the active sensitization to Der p has also induced a hypersensitivity of the airways.

Indeed, for a given value of Penh, the necessary MCh concentrations have been reduced, in the sensitized rats compared to the 2 other groups.

Lambert et al [13] have observed in sensitized rats with Der p a bronchoconstriction and a hyper responsiveness to the Acetycholine (Ach). In the same way the exhibition to the histamine of sensitized guinea pigs by intraperitoneal injection with the ovalbumin (OV) gave a bronchial hyperreactivity [14; 15] have shown that the exhibition to the histamine induces a bronchial hyperreactivity (BHR). This bronchial hyperreactivity appears 6 hours after the test of provocation with the OV or the 5'-AMP. It is preserved 24 hours at the same time as the significant increase of the rate of eosinophil and macrophages.

Persistent hyper responsiveness of more than 4 days has been observed by Chung et. al. [16] in sensitized dogs and by Gundel et al. [17] on primates. Repeated
inhalation of allergen in these primates was characterized by a bronchoconstrictive reactivity dependent-dose to the MCh, hypersensitivity with deviation of curves toward the left and a bronchial hyperreactivity characterized by the decrease of the necessary metacholine dose to induce an increase of resistances of the VA. In a similar way in our asthmatic rats we observed in vivo a hyperresponsiveness and airways hypersensitivity. On the rings of trachea, EPB and IPB, the CCh has induced a variable contractile response in accordance with the experimental conditions and the position of the tissue along the bronchial tree. Indeed the contractile response in the sensitized rats was more important than the one of control and witness rats. On the other hand, it was more important on the IPB that on the trachea and the EPB. The effect of the active sensitization not only resulted in a non specific hyperresponsiveness to the CCh on rings of rats sensitized and a displacement of the curve toward the left. Other authors like Gerber et al., [18] had already demonstrated that the stimulation of rodent trachea sensitized by heterologous proteins led to a specific response to the allergen. In control rats we observed a hyper-responsiveness and hypersensitivity compared to rats witnesses. It was in favor of a non specific stimulation of the immune system with the Al2O3.

V. CONCLUSION

The active sensitization with the allergenic proteins from of Dermatophagoides pteronyssinus has induced a significant increase of the inflammatory cell density mainly of eosinophil count, macrophages, lymphocytes and mast cells. Norway brown rats actively sensitized present a specific Der p extract and a non specific response dependent-concentration to the muscarinic agonists. Indeed active sensitization of brown Norway rats to Der P extracts induced a hyperresponsiveness and hypersensitivity of airways. The type of animal and the way of exhibition are some important elements to get an experimental animal model of which features of the asthma are close those of man.

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REFERENCES