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Expression of Th-2 cytokines interleukin-4 and -5 and of Th-1 cytokine interferon-γ in ovalbumin-exposed sensitized Brown-Norway rats

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SUMMARY

We determined the expression of Th-2 type cytokines, interleukin-4 (IL-4) and IL-5, and of the Th-1 type cytokine, interferon-γ (IFN-γ), in the Brown-Norway rat. Rats were intraperitoneally sensitized with ovalbumin and 21 days later were either exposed to ovalbumin or saline aerosol. The value $-\log PC_{300}$ (PC_{300} = concentration of acetylcholine needed to increase baseline lung resistance by 300%) was 2·49 ± 0·15 in sensitized, exposed rats, was higher than in sensitized, saline-exposed or naive rats (1·54 ± 0·27 and 1·63 ± 0·06 respectively, $P < 0·05$). There was a significant increase in eosinophils in bronchoalveolar lavage fluid and in airway submucosal airway tissues in the sensitized exposed group. Reverse-transcriptase polymerase chain reaction was performed on total lung RNA using primers for IL-4, IL-5, IFN-γ and β-actin. IL-4 and IL-5 mRNA levels in control and sensitized saline-exposed rats were not detectable, but increased levels were found in sensitized and ovalbumin-exposed rats with levels of 0·25 ± 0·01 and 0·98 ± 0·02% of β-actin mRNA as assessed by densitometric measurements. Expression of IFN-γ mRNA was significantly reduced in sensitized and ovalbumin-exposed rats. As in asthmatic airways, there is an increased expression of Th-2 cytokines, IL-4 and IL-5, together with a reduction in the Th-1 cytokine, IFN-γ, thus supporting a role for Th-2 cytokines in allergic eosinophilic inflammation.

INTRODUCTION

Recent studies in patients with mild allergic asthma have demonstrated the presence of eosinophil and T-lymphocyte infiltration and activation in the airways submucosa[1–3] and have led to the hypothesis that T cells expressing a repertoire of cytokines of the Th-2 but not of the Th-1 phenotype may orchestrate allergic airway inflammation.[3,4] In order to obtain direct evidence for an involvement of T lymphocytes in bronchial hyperresponsiveness and allergic eosinophilic inflammation, appropriate animal models have been studied.[5] The sensitized Brown-Norway rat demonstrates many features similar to human allergic asthma. Following allergen challenge, an early and late phase reaction has been described,[6] with the onset of airway hyperresponsiveness,[7] accumulation of inflammatory cells including eosinophils and T lymphocytes in the airways,[8,9] and an increase in the number of activated T lymphocytes as measured by the number of cells bearing the $CD25^+$ marker in bronchoalveolar lavage fluid.[9] To examine further the potential for T cells in our model of bronchial hyperresponsiveness and eosinophilic inflammation, we investigated changes in cytokine pattern produced 24 hr after ovalbumin-aerosol exposure in sensitized Brown-Norway rats.

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MATERIALS AND METHODS

Animals, sensitization procedures and allergen exposure

Virus-free inbred male Brown-Norway rats (Harlan Olac Ltd, Bicester, UK; 230–250 g weight) were injected with ovalbumin (OA; 1 ml, 1 mg/ml) in $Al(OH)_3$ (100 mg/ml) in 0·9% saline intraperitoneally (i.p.) on 3 consecutive days. Aerosol exposure was accomplished by placing the rats in a 6·5-l plexiglass chamber connected to a DeVilbiss PulmoSonic nebulizer (model No. 2512, DeVilbiss Health Care, UK, Ltd., Feltham, Middlesex, UK) that generated an aerosol mist pumped into the exposure chamber by the airflow supplied by a small animal ventilator set at 60 strokes/min with a pumping volume of 10 ml.

We studied three groups of rats.

(1) *Naive animals*: non-sensitized and non-exposed animals were kept in identical conditions to the sensitized animals for 3 weeks and studied thereafter at the same time-points ($n = 9$).

(2) *Sensitized and saline-exposed animals*: 1 ml of a 1 mg OA/100 mg $Al(OH)_3$ in 0·9% (wt/vol) saline suspension was injected i.p. for 3 consecutive days. Twenty-one days after the final injection, rats were exposed to saline aerosol for 15 min ($n = 7$). Animals were then studied 18 to 24 hr after exposure.

(3) *Sensitized and OA-exposed animals*: 1 ml of a 1 mg OA/100 mg $Al(OH)_3$ in 0·9% (wt/vol) saline suspension was

injected intraperitoneally for 3 consecutive days. Twenty-one days after the final injection, animals were exposed to 1% OA aerosol for 15 min ($n = 6$) and then studied 18 to 24 hr later. In all rats, bronchoalveolar lavage and collection of lung tissues were made at 20–24 hr after they were exposed to OA aerosol.

Measurement of airway responsiveness

Airway responsiveness was measured as previously described elsewhere.[8] Briefly, anaesthetized, tracheostomized and ventilated rats were monitored for airflow, transpulmonary pressure and blood pressure. Lung resistance was simultaneously calculated using a software program (LabView, National Instruments, Austin, TX, USA). Increasing half $\log_{10}$ concentrations of acetylcholine were administered by inhalation (45 breaths) with the initial concentration set at 10^{-4} mol/l. The challenge was stopped when an increase in lung resistance exceeding 600% over the initial baseline was obtained. The concentration of acetylcholine needed to increase baseline lung resistance by 300% (PC_{300}) was determined from the log concentration–lung resistance curve by linear interpolation.

Bronchoalveolar lavage and cell counting

Lungs were lavaged with 20 ml of total volume of sterile saline after measurement of airway responsiveness. Differential cell counts were made from cytospin preparations stained by May–Grünwald stain. Cells were identified by standard morphology and 500 cells were counted under ×400 magnification.

Collection of lung tissues and immunohistochemistry

The thoracic cavity was opened and the lungs were removed and inflated with 5 ml saline/OCT (1:1). Blocks (5 mm^3) of the left lung tissue around the main bronchus were cut, embedded in OCT medium (Raymond A. Lamb, London, UK) and snap-frozen using isopentane (BDH, Poole, Dorset, UK) in liquid nitrogen. Cryostat sections (6 μm) of the tissues were cut, air-dried and fixed in absolute alcohol. Fixed slides were air-dried, wrapped in aluminium foil and stored at −80° until used.

We have used the mouse monoclonal antibody directed against human major basic protein, BMK-13,[10] for the detection of rat eosinophils. This monoclonal antibody gives more sensitive and specific staining of rat eosinophils in frozen sections, when compared with carbol chromotrope 2R or haematoxylin and eosin staining. Cryostat sections of bronchial and lung tissue were incubated with BMK-13 at a dilution of 1:30 for 30 min at room temperature. After labelling with the second antibody layer, rabbit anti-mouse IgG, positive staining was visualized by the alkaline phosphatase–anti-alkaline phosphatase technique. Specifically-bound alkaline phosphatase was detected as a red colour following incubation with Naphthol AS-MX phosphate in 0·1 M trismethylamine–HCl buffer (pH 8·2) containing levamisole to inhibit endogenous alkaline phosphatase and 1 mg/ml Fast Red–TR salt. Sections were counterstained with Harris haematoxylin (BDH Ltd) and mounted in Glycergel (DAKO Ltd, High Wycombe, UK). System and specificity controls were carried out on all staining runs. Slides were read in a blind fashion and in coded random order by two observers using an Olympus BH2 microscope (Olympus Optical Company Ltd, Tokyo, Japan). The inter-observer variability was < 10%. The submucosal area for counts was quantified by use of a computer-assisted graphics tablet visualized by a sidearm attachment to the microscope. Counts were expressed as positive cells per mm^2 of cross-sectional subepithelial surface.

Isolation of total RNA from lung tissue

Lung tissue pieces were finely minced with cooling in liquid nitrogen (BOC, Luton, UK), immediately suspended in 15 ml complete D solution (distilled water, 0·75 M sodium citrate (pH 7), 10% sarcosyl (Sigma, Poole, UK); complete D solution was made by adding 0·36 ml 2-mercaptoethanol to 50 ml of stock solution) and homogenized by a Polytron homogenizer. A volume of 1·5 ml of 2 M sodium acetate (Sigma) (pH 4·0), 15 ml of phenol (water saturated) (Sigma) and 3 ml of chloroform/isoamyl alcohol mixture (49:1) (Camlab Ltd, Cambridge, UK) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 min. The suspensions were centrifuged at 14 500 *g* (12 000 r.p.m.) for 15 min at 4°. The aqueous phase (RNA) was transferred to a fresh tube, mixed with 15 ml of isopropanol (BDH) and then stored at −20° for at least 1 hr. Tubes were centrifuged as above and the resulting pellets were resuspended in 5 ml of complete D solution. Samples were then reprecipitated with 5 ml of isopropanol at −20° overnight. The tubes were then centrifuged (14 500 *g* for 15 min at 4°) and the final pellet was resuspended in 5–10 ml 75% ethanol. The samples were centrifuged (14 500 *g* for 15 min at 4°) and freeze-dried (15 min) and finally dissolved in 1 ml of RNAase-free water (Sigma). Measurement of RNA was performed by adding 10 ml of the above mixture to 990 ml of distilled water. Optical density was measured at 260 nm in a spectrophotometer to calculate yield. Each sample was analysed on a 1·5% agarose gel to check for degradation. RNA samples were stored at −20°.

Preparation of cDNA

RNA (4·0 μg) from each sample was reverse-transcribed in 40 ml reverse transcriptase buffer containing 5 mg oligo-dT (Pharmacia, Uppsala, Sweden), 20 U AMV reverse transcriptase, 2·25 mM dNTP, 0·1 mg/ml bovine serum albumin (BSA), 10 mM dithiothreitol and 40 U RNAguard. A reagent mix was made up for each batch of samples and an equal volume added to each. Reactions were made up to 40 ml by adding 0·625% RNAguard, 2·5 mM DTT. Tubes were incubated at room temperature for 20 min and 30 min at 42° before storage at −20°.

Analysis of mRNA by semi-quantitative polymerase chain reaction

Polymerase chain reaction (PCR) reactions consisted of 5 μl reverse transcribed RNA in 40 ml reverse transcriptase buffer containing 1·25 mM of each primer, 0·3 ml 25 mM dNTP, 0·35 U Perfect Match polymerase enhancer and 1·2 U AmpliTaq polymerase. A mixture of reagents was made up for each batch of samples on ice, and 35 ml was added to each 5 ml sample in a 0·5 ml microtube. Positive controls consisted of PCR-generated fragments which were diluted to give an appropriate number of copies per tube ($2{\cdot}5 \times 10^4$–$5{\cdot}0 \times 10^6$). For interleukin-4 (IL-4) PCR, plasmid pRIL was used as a positive control. Reaction mixtures were whirlmixed, centrifuged briefly and overlaid with one drop of mineral oil. Samples were centrifuged briefly again and transferred to a thermal

cycler at 72°. After an initial denaturing step (3 minutes at 94°), up to 29 PCR cycles were performed, each consisting of three steps: denaturing step, 1 min 94°, annealing step, 60° for 2 min (or 15 min for the first four cycles), primer extension step: 2 min 72°. Aliquots (6 ml) were taken from each sample after two or three different numbers of cycles. Aliquots were mixed with an equal volume of 2× sample buffer and run on 3·2% agarose gels in glycine buffer stained with ethidium bromide. pBR322/MspI or similar marker fragments (250 ng) were run in duplicate on each gel. Photographs were taken under ultraviolet illumination on Agfa APX25 film and the negatives were scanned on a Molecular Dynamics Image Quant™ (Boston, MA) densitometer. Densities of marker fragments were plotted against molecular weight and used to estimate yields of PCR target fragments. Copies of mRNA per mg total RNA were calculated as described.[11] Results were expressed as a percentage of β-actin mRNA copies.

Data analysis

Data have been presented as mean ± SEM. PC_{300} values are presented as $-\log_{10} PC_{300}$. The Kruskal–Wallis test for analysis of variance was used for multiple comparisons of the three groups studied. For comparison of individual groups, the Mann–Whitney test was used. For regression analysis, we pooled the groups after testing the homogeneity of variances using Bartlett's test. Data were analysed with MINITAB standard statistical package (Minitab Inc., State College, PA, USA). A P value of $< 0{\cdot}05$ was accepted as significant.

RESULTS

Bronchial responsiveness

Mean PC_{300} was 2·49 ± 0·15 in OA-exposed sensitized rats which was significantly higher than in saline-exposed sensitized rats and in naive rats (PC_{300} were 1·54 ± 0·27 and 1·63 ± 0·06, respectively; $P < 0{\cdot}05$; Fig. 1), indicating an increase in airway responsiveness.

BAL cell profile and tissue eosinophils

When compared with saline-exposed sensitized rats, OA-exposed rats had significantly increased numbers of eosinophils, neutrophils and lymphocytes ($P < 0{\cdot}002$; $P < 0{\cdot}003$ and $P < 0{\cdot}05$ respectively) (Fig. 2(a)). Thus, total eosinophil counts in naive and saline-exposed sensitized rats were $70{\cdot}9 \pm 0{\cdot}7 \times 10^4$ and $2{\cdot}9 \pm 1{\cdot}5 \times 10^4$ respectively, compared to $52{\cdot}2 \pm 5{\cdot}2 \times 10^4$ in OA-exposed and sensitized rats ($P < 0{\cdot}002$). There were no significant differences in the numbers of macrophages and in total cell counts between the groups.

The number of eosinophils staining with BMK-13 monoclonal antibody in airway submucosa was significantly increased in sensitized OA-exposed rats (177·2 ± 105·0 compared to 9·2 ± 4·5 and 5·8 ± 3·7 in saline-exposed sensitized rats and naive rats respectively; $P < 0{\cdot}001$; Fig. 2(b)). Sensitization alone had no significant effect.

Cytokine mRNA profile using RT–PCR

Following 29 cycles of PCR, there was no detectable mRNA transcripts for IL-4 and IL-5 in the lungs of the naive and

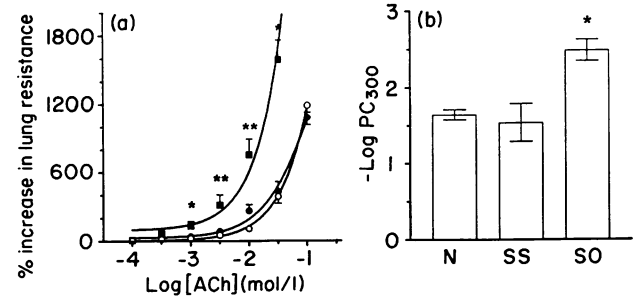

Figure 1. (a) Mean increase (%; ±SEM) in lung resistance above baseline values following aerosol challenge with increasing concentrations of acetylcholine (ACh) in non-sensitized/non-exposed rats (open circles), OA-sensitized/saline-exposed rats (filled circles) and OA-sensitized/OA-exposed rats (filled squares); * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$ between sensitized/saline-exposed and sensitized/OA-exposed groups. (b) Mean $-\log PC_{300}$ to acetylcholine (±SEM) in Brown-Norway rats. N, non-sensitized/non-exposed rats; SS, OA-sensitized/saline-exposed rats; SO, OA-sensitized/OA-exposed rats. Statistical significance as for panel (a).

sensitized but saline-exposed rats (Fig. 3). However, in sensitized and OA-exposed rats, IL-4 mRNA transcripts increased in four out of six rats and IL-5 mRNA transcripts in all six rats ($P < 0{\cdot}0001$). Interferon-γ (IFN-γ) mRNA transcripts were expressed at a relatively high level (1·06 ± 0·002% of β-actin mRNA), compared to IL-4 and IL-5 mRNA transcripts. Sensitized rats exposed to saline showed a significant decrease in IFN-γ mRNA transcripts to 0·79 ± 0·05% ($P < 0{\cdot}002$), but this was not significantly different from sensitized and OA-exposed rats.

Relationships between airway responsiveness, eosinophilia and cytokine mRNA

There was a positive correlation between the numbers of

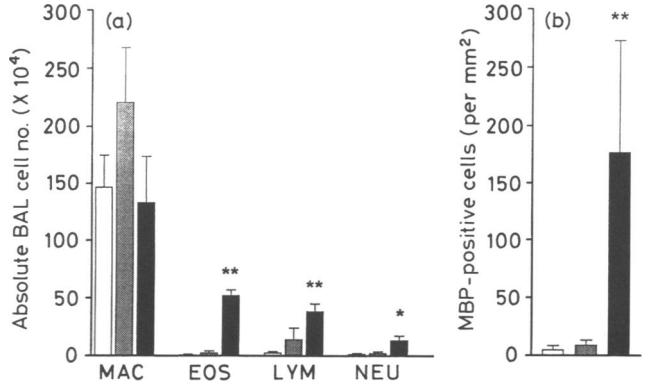

Figure 2. Mean (±SEM) numbers of macrophages (MAC), eosinophils (EOS), neutrophils (NEU) and lymphocytes (LYM) recovered from bronchoalveolar lavage fluid in non-sensitized/non-exposed rats (open bar), OA-sensitized/saline-exposed rats (cross-hatched bar) and OA-sensitized and OA-exposed rats (solid bars); * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$ between sensitized/saline-exposed and sensitized/OA-exposed groups. (b) Mean (±SEM) of submucosal eosinophils as assessed by cells labelled by an antibody to major basic protein (MBP) in the same three groups of rats as in (a). Legends and statistical differences are the same as those for (a).

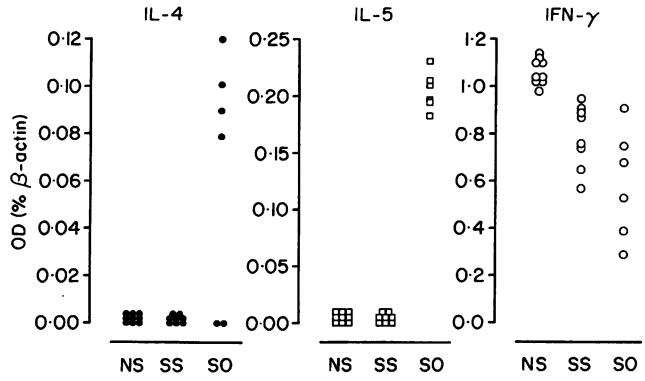

Figure 3. Individual optical densities for IL-4, IL-5 and IFN-γ mRNA expression assessed by reverse-transcription polymerase chain reaction expressed as a percentage of β-actin mRNA in lung tissues. NS, non-sensitized-non-exposed rats; SS, OA-sensitized/saline-exposed rats; SO, OA-sensitized/OA-exposed rats.

Table 1. Correlations between airway responsiveness, eosinophilia and cytokine mRNA expression

	IL-4 mRNA	IL-5 mRNA	IFN-γ mRNA
$-\log PC_{300}$			
correlation (r)	0·55	0·71	−0·60
P value	< 0·01	< 0·001	< 0·003
BAL eosinophil			
correlation (r)	0·75	0·95	−0·68
P value	< 0·001	< 0·001	< 0·001
Tissue eosinophil			
correlation (r)	0·52	0·57	−0·51
P value	< 0·05	< 0·005	< 0·02

eosinophils in BAL and MBP^+ cells in the mucosal tissue ($r = 0{\cdot}44$; $P < 0{\cdot}05$) and between the numbers of BAL eosinophils and $-\log PC_{300}$ ($r = 0{\cdot}73$; $P < 0{\cdot}001$), when data were pooled for all rats. The relative amounts of both IL-4 and IL-5 mRNA transcripts showed a significant positive correlation with BAL and tissue eosinophilia, and $-\log PC_{300}$ and IFN-γ mRNA transcripts showed an increase correlation with all these parameters (Table 1).

DISCUSSION

Exposure of OA-sensitized rats or OA aerosol induced a quantitative increase in mRNA for IL-4 and IL-5 in the lung. IL-4 and IL-5 mRNA were not detectable in naive animals but were present in four and six out of the six rats that were sensitized and exposed to OA, respectively. These changes were not secondary to sensitization itself since sensitization and exposure to saline did not result in significant changes in the mRNA for IL-4 and IL-5. In contrast, IFN-γ mRNA was expressed in all naive animals and fell significantly after sensitization alone, with OA exposure not leading to any further significant decrease in IFN-γ mRNA. We also confirmed the increase in bronchial responsiveness to acetylcholine in the sensitized ovalbumin-exposed rats, associated with an eosinophilic influx in airway submucosal tissues and with an increase in eosinophil, neutrophil and lymphocyte counts in bronchoalveolar lavage fluid.[8,9]

More detailed characterization of the T lymphocyte has been reported in previous studies of the Brown-Norway rat model.[7,9,12] In bronchoalveolar lavage fluid, allergen exposure of OA-sensitized Brown-Norway rats resulted in an increase in the numbers of $CD4^+/CD25^+$ activated T cells as measured by flow cytometric analysis.[7] In the airway submucosa, there was a significant increase in the number of $CD8^+$ and $CD25^+$ T cells at 24 hr after allergen exposure, with the increase in the number of $CD8^+$ and $CD25^+$ T cells correlating significantly with the numbers of these T cells and eosinophils.[9] However, the numbers of $CD4^+$ T cells were not significantly altered. Direct evidence for the capacity for T cells to effect directly bronchial hyperresponsiveness and eosinophilic inflammation was obtained by adoptive transfer of spleen T cells obtained from ovalbumin-sensitized donor rats. Thus, T cells, in particular $CD4^+$ T cells, induced bronchial hyperresponsiveness and submucosal eosinophilic inflammation,[13,14] thus directly implicating the T cell.

Our data support a role for the Th-2-derived cytokines, IL-4 and IL-5, in the induction of bronchial hyperresponsiveness and eosinophilic inflammation in our model. We found a significant correlation between bronchial responsiveness and the level of IL-4 and IL-5 mRNA transcripts measured in the lung. Since there was concomitant expression of IL-4 and IL-5 genes, it seems likely that an expansion of Th-2-like cells had occurred. In addition, other cell types, such as eosinophils and mast cells, may express these cytokines. IL-5 has been shown to cause selective eosinophil (as opposed to neutrophil) recruitment[15] and activation[16] and is the only known cytokine that can promote terminal differentiation of eosinophil precursors[17] and enhance survival.[18] IL-4 can also contribute to selective eosinophil accumulation in sites of allergic inflammation.[19] A blocking antibody to either IL-4 or IL-5 has been shown to inhibit OA-induced airway eosinophilia and airway hyperresponsiveness.[20,21] Finally, eosinophil granule major basic protein can induce airway hyperresponsiveness in the rat *in vivo*,[22] thus linking eosinophils to the induction of airway hyperresponsiveness. Both IL-4 and Il-5 could be primarily involved in the bronchial hyperresponsiveness and eosinophilic inflammation observed in our Brown-Norway rat model but direct evidence is not currently available.

The presence of high levels of IFN-γ mRNA in comparison to undetectable levels of IL-4 and IL-5 indicates that in the naive Brown-Norway rat there is a preponderance of Th-1-like cells. The presence of different cytokines during T-cell differentiation appears to be at least one of the factors that determine the functional phenotype of mature T cells. IFN-γ favours the development of Th-1-like cells and inhibits the growth of Th-2 cells.[23,24] In mixed rat spleen cell culture, mitogenic stimulation favoured differentiation of naive rat T cells into effector cells expressing a Th-1 not a Th-2 cytokine profile. The addition of IL-4 to activated $CD4^+$ cell cultures induces cells that secrete high levels of Th-2 cytokines,[25,26] and has been shown to stimulate preferentially the growth of Th-2-like cells.[24,27] In rat splenocytes, IFN-γ inhibits IL-4 production and suppresses the generation of IL-4-responsive T cells.[28] The level of IFN-γ mRNA in the lung was reduced after sensitization with OA, an observation akin to that of stimulated purified spleen T cells of

immunized rats producing less IFN-γ than those of non-immunized rats.[29] Whether reduced expression of IFN-γ may be involved in bronchial hyperresponsiveness and eosinophil influx is not known, but our data would suggest that the reduction in IFN-γ mRNA resulted from the sensitization procedure *per se*.

The Brown-Norway rat appears to be a suitable model for unravelling the role played by the T lymphocyte in bronchial hyperresponsiveness and eosinophilic inflammation. The induction of Th-2 cytokines, IL-4 and IL-5, following allergen challenge as has been described in human allergic asthmatics after allergen challenge[30] further supports the usefulness of this model. The Brown-Norway rat model can be used to dissect out the mechanisms by which T lymphocytes induce lung eosinophilia and bronchial hyperresponsiveness.

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