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The effect of prolonged exposure to NO_2 from birth on airways responsiveness in rabbits sensitized at birth

G.J. Douglas*, J.F. Price**, C.P. Page*

The effect of prolonged exposure to NO_2 from birth on airways responsiveness in rabbits sensitized at birth. G.J. Douglas, J.F. Price, C.P. Page. ©ERS Journals Ltd 1995.

ABSTRACT: Our aim was to determine whether daily exposure to 4 ppm nitrogen dioxide (NO_2) from birth until 3 months of age influenced the development of airways hyperresponsiveness and atopic sensitivity in immunized rabbits.

Littermate New Zealand White (NZW) rabbits were immunized within 24 h of birth by *i.p.* injection of house dust mite antigen in $Al(OH)_3$ gel, and exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1}, 5 days·week^{-1}. At 3 months, bronchoalveolar lavage (BAL) and serum samples were obtained. Airways responsiveness was measured as the provocative concentrations (mg·ml^{-1}) of histamine or methacholine required to elicit a 50% increase in airway resistance (R_LPC_{50}) and a 35% decrease in dynamic compliance ($CdynPC_{35}$).

There were no differences in total cell or differential cell counts recovered in BAL fluid between control and NO_2 exposed animals. Airways responsiveness did not differ between groups of animals (histamine R_LPC_{50} values: air (n=15) *versus* NO_2 (n=13), respectively, 9.98±1.32 *versus* 16.43±1.45 mg·ml^{-1}; $CdynPC_{35}$ values: 16.60±1.44 *versus* 14.95±1.43 mg·ml^{-1}; methacholine R_LPC_{50} values: air (n=14) *versus* NO_2 (n=12), respectively, 2.18±1.51 *versus* 2.21±1.32 mg·ml^{-1}; $CdynPC_{35}$ values: 2.64±1.41 *versus* 2.85±1.31 mg·ml^{-1}). There was no difference in sensitization between groups of animals exposed to air or NO_2, evaluated either by cutaneous responsiveness to intradermal antigen, or serum immunoglobulin E (IgE) levels assessed by the passive cutaneous anaphylaxis (PCA) reaction.

We conclude that daily inhalation of 4 ppm NO_2 during the first three months of life does not affect airways responsiveness and atopic status of rabbits sensitized at birth. The lack of influence of NO_2 in this model may be related to the *i.p.* route of immunization.

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*Dept of Pharmacology, King's College, University of London, London, UK. **Dept of Thoracic Medicine, King's College Hospital, London, UK.

Correspondence: G.J. Douglas
Dept of Pharmacology
King's College
Manresa Road
London SW3 6LX
UK

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Over the past decade, a number of studies from several countries have shown that the incidence of atopy and asthma is increasing, particularly amongst children, but the cause of this rise remains unknown [1, 2]. Together with a genetic predisposition for allergy, some environmental factor is thought to influence the development of asthma and other allergic disorders [3]. An association between air pollutants and the incidence of respiratory disease and asthma has been reported in a number of epidemiological studies, and there is strong evidence that asthma is more frequent and severe in polluted urban areas as opposed to unpolluted rural areas [1, 4, 5], but there is some controversy whether outdoor air pollution influences the prevalence of asthma. Efforts to reduce sulphur dioxide (SO_2) and black smoke emissions in Western countries have not resulted in a decrease in asthma. On the contrary, recent reports suggest that there is more asthma and hayfever in cities located in the former West Germany compared to former East German cities, that had far greater levels of SO_2 and particulates prior to reunification of the country [6, 7]. However, during the same period, nitrogen dioxide (NO_2) levels were higher in the West German cities than those in East Germany [7].

Nitrogen dioxide is a pollutant gas present both in the outdoor environment, resulting from emissions from automobile exhausts, and the indoor environment, from gas fires and cookers, which has been associated with an increased morbidity rate for respiratory disease in children [1, 4]. A number of actions implicate NO_2 as a co-factor in the development of asthma. In laboratory animals, NO_2 inhalation decreases respiratory clearance rates [8, 9], inhibits alveolar macrophage function [10, 11], and alters subpopulations of T-lymphocytes [12, 13].

Over 60% of asthma at any age has its origins in childhood, and most severe childhood asthma has its origins in infancy. It is now well-recognized that the initial contact with an allergen early in life, before the immune system has reached full competency, will determine later sensitivity to that allergen [14]. In rabbits, immunization within 24 h of birth with an antigen together with the adjuvant $Al(OH)_3$ induces the preferential

production of antigen specific immunoglobulin E (IgE) antibodies [15]. Animals sensitized neonatally in this way exhibit several features of the asthmatic. They undergo early and late airways responses following antigen challenge [16–18], which is associated with airways oedema and inflammatory cell infiltration [19, 20], are hyperresponsive to inhaled histamine and methacholine compared with naive rabbits [21, 22], and exhibit airways hyperresponsiveness following challenge with either antigen or platelet-activating factor [18–21]. Repeated measurements of lung function are readily made in the rabbit, and each animal may be used as its own control [22–24]. We have, therefore, chosen the rabbit as the species in which to conduct our experiments investigating the effects of exposure to pollutant gases from birth.

Thus, the environmental pollutant NO_2 not only has direct effects on the airways, but additionally has been implicated as an adjuvant factor in the development of atopy and asthma [25, 26]. Nonetheless, the precise contribution of NO_2 and other pollutant gases to the development of atopy or bronchial hyperresponsiveness remains unknown. In this study, we asked whether exposure to NO_2 during the first three months of life increases airways hyperresponsiveness and sensitization in rabbits immunized against the house dust mite (HDM) *Dermatophagoides pteronyssinus*.

Materials and methods

Animals

New Zealand White rabbits were supplied by Froxfield Farms (Petersfield, Hampshire, UK). Naive male rabbits (2.0–3.0 kg) and neonatal littermates of either sex were used. In the prolonged study, littermate rabbits of both sexes were immunized, using the protocol outlined below, and exposed to air or NO_2 at the breeders' unit before transfer to our laboratory at 3 months of age. Animals were housed under identical conditions when not in the exposure chamber. Littermates were held together with their dame until weaning at 3 weeks. All procedures described were subject to Home Office approval and were performed under the Animals (Scientific Procedures) Act 1986.

Study design

After immunization, an equal number of pups from each litter were randomly assigned to undergo exposure to either ambient air or NO_2 (4 ppm) for 2 h·day^{-1}. Data were obtained in this study from 29 rabbits originating from five separate litters. Prolonged exposure studies were performed on immunized rabbits from the day of birth. At 3 months of age, bronchoalveolar lavage (BAL) and serum samples were obtained on the day following the last exposure. Measurements of airways responsiveness to the bronchoconstrictors histamine and methacholine were made 3–8 days after the final air or gas exposure. Two parameters of allergic sensitization were measured: the immediate inflammatory response in the skin was assessed, and indices of serum IgE levels were determined using the homologous passive cutaneous anaphylaxis (PCA) reaction in naive rabbits.

Materials and drugs

Materials and drugs were purchased from the following sources: nitrogen (BOC Ltd, London, UK); mixtures of 10% NO_2 in air (BOC Ltd, Special Gases, Guildford, Surrey, UK); sterile aluminium hydroxide $Al(OH)_3$ gel (Rehydragel; Reheis, Dublin, Eire); sterile pyrogen free 0.9% sodium chloride solution (saline; Baxter Health Care, Thetford, Norfolk, UK); lyophilized allergen extract of *Dermatophagoides pteronyssinus* (Aquagen, ALK [503]; vial number 4: 100,000 SQ-U·ml^{-1} batch No. 2556/236154; ALK Allergologisk Laboratorium A/S, Hørsholm, Denmark); chromotrope 2R, sterile Dulbecco's phosphate buffered saline (PBS), Evans blue dye, haematoxylin, histamine diphosphate, methacholine hydrochloride and sodium pentobarbitone (Sigma Chemical Co., Poole, Dorset, UK); diazepam (Roche Products Ltd, Welwyn Garden City, Hertfordshire, UK); Hypnorm (a mixture of fentanyl citrate, 0.315 mg·ml^{-1}, and fluanisone, 10 mg·ml^{-1}) (Janssen Pharmaceutical Ltd, Grove, Oxfordshire, UK).

Pulmonary function measurements

Measurements of pulmonary function were made in spontaneously breathing 3 month old rabbits, premedicated with diazepam (2.5 mg·kg^{-1}, *i.p.*) and anaesthetized with Hypnorm (0.4 ml·kg^{-1}, *i.m.*), using a modification of the method described previously [23]. Animals were intubated with a 3.0 mm cuffed endotracheal tube, and a latex oesophageal balloon was inserted into the lower third of the oesophagus. Measurements of flow were obtained by attachment of the endotracheal tube to a heated (37.5°C) Fleisch pneumotachograph connected to a differential pressure transducer. The oesophageal balloon was attached to a second differential pressure transducer open to air, from which values of transpulmonary pressure (Ptp) were derived. Using an on-line respiratory analyser (PMS Version 4.0; Mumed Ltd, London, UK), values for lung resistance (R_L) and dynamic compliance (Cdyn) were calculated from measurements of flow and Ptp [23].

Measurement of airways responsiveness

Airways responsiveness was measured in response to inhaled histamine or methacholine using minor modifications of the method described previously [23]. Agents were aerosolized in an ultrasonic nebulizer and administered directly into the lungs *via* the endotracheal tube. After measurement of basal lung function parameters,

animals were administered saline as an aerosol for 2 min, as a baseline with which to compare responses to the bronchoconstrictors, and lung function measurements were again made. Doubling concentrations of either histamine (1.25–80 mg·ml^{-1}) or methacholine (0.31–20 mg·ml^{-1}) aerosols were then cumulatively administered for 2 min periods. Lung function parameters were recorded either immediately (following histamine challenge), or after a 2 min period of equilibration (following methacholine challenge). The provocation concentrations (PC) of histamine or methacholine that produced a 50% increase in R_L (R_LPC_{50}) and a 35% decrease in Cdyn ($CdynPC_{35}$) were determined for individual animals by linear interpolation, and used as indices of airways responsiveness.

Bronchoalveolar lavage (BAL) and serum samples

BAL samples were obtained from intubated rabbits anaesthetized with diazepam/Hypnorm, 1 day after the last exposure to air or gas. A polyethylene cannula was passed down the endotracheal tube until it was wedged gently against the airway wall. Saline (5.0 ml) was instilled into, and immediately aspirated from, the lungs under vacuum and collected in a polystyrene tube. The numbers of cells recovered were determined, and after staining with Lendrum's stain differential cell counts were made [23]. Blood samples (approx. 6 ml) were taken from the marginal ear vein into glass phlebotomy tubes (Monoject Z/10) at the same time that BAL was performed, kept at room temperature for 1 h and stored overnight at 4°C. Serum was aspirated from the clot, centrifuged to remove debris, aliquoted and stored at -20°C until required for PCA tests.

Immunization to house dust mite antigen

Rabbits were immunized with an extract of *Dermatophagoides pteronyssinus* in $Al(OH)_3$ gel [27]. HDM antigen (0.5 ml; 100,000 SQ-U·ml^{-1}) was mixed with $Al(OH)_3$ moist gel (0.5 ml) and saline (1.0 ml). Each rabbit received 0.5 ml of this antigen-adjuvant mixture within 24 h of birth by the *i.p.* route. The injection was repeated weekly for the first month of life, and then biweekly until 13 weeks of age.

Exposure to NO_2

The environmental exposure chamber system employed has been described previously [27]. Laboratory air was drawn through a large (0.71 m^3) stainless steel and glass chamber, which can hold a litter of rabbits up to 3 months of age, by a centrifugal fan at a rate of 0.42 m^3·min^{-1} (25 m^3·h^{-1}), equivalent to 35 complete air changes per hour. Cylinder NO_2 gas was diluted in the incoming air stream to the desired concentration. The gas level within the chamber was continuously monitored throughout the exposure period by an electrochemical gas diffusion sensor, displayed on a digital readout, and recorded on a flatbed pen recorder. After rapid equilibration, the gas concentration within the chamber was reliably maintained for at least 8 h [27].

Measurement of cutaneous sensitivity to HDM

Direct skin tests to HDM antigen were performed in immunized rabbits following completion of lung function measurements. Rabbits were anaesthetized by an *i.v.* injection of sodium pentobarbitone (30 mg·kg^{-1}) *via* an indwelling cannula (23 gauge Butterfly; Abbot Ireland Ltd, Sligo, Eire) in the marginal ear vein. The dorsal skin hair was closely clipped and a balanced site pattern marked either side of the midline. Evans blue dye (10 mg·kg^{-1}, 2.5% in saline) was administered *i.v.* to aid visualization of the inflammatory response. Ten minutes later serial twofold dilutions of HDM antigen (0.3–80 SQ-U·site^{-1}), or PBS as the control, were injected intradermally (0.1 ml) in four replicates. The rabbits were killed 60 min after *i.d.* injection with an overdose of sodium pentobarbitone (60 mg·kg^{-1} *i.v.*) and the back skin was removed. The titration end-point was taken as the concentration of HDM antigen that caused a greater response in at least two replicates than that of the control when viewed from the underside.

Passive cutaneous anaphylaxis reactions

An index of the serum IgE levels from immunized rabbits was obtained by titration of immune sera samples against a nonimmune serum in the passive cutaneous anaphylaxis (PCA) test. Recipient naive male rabbits (2.0–3.0 kg) were anaesthetized with Hypnorm (0.4 ml·kg^{-1} *i.m.*) and the dorsal skin hair was closely clipped. A pattern of six blocks of nine sites for 54 *i.d.* injections was marked on either side of the midline. Serial twofold dilutions (between 1:2–1:512) in PBS were made of eight test sera and one nonimmune serum, and injected (0.1 ml) *i.d.* into three recipient rabbits. After a 72 h fixation period, the recipient rabbits were reanaesthetized with sodium pentobarbitone (30 mg·kg^{-1} *i.v.*) *via* an indwelling cannula (23 gauge Butterfly) in the marginal ear vein. Evans blue dye (10 mg·kg^{-1}) was administered *i.v.* followed 10 min later by *i.v.* HDM antigen (5,000 SQ-U·kg^{-1}). Thirty minutes after antigen challenge, the rabbits were killed with an overdose of sodium pentobarbitone (60 mg·kg^{-1} *i.v.*) and the back skin removed. The titration end-point was taken as the highest dilution at which the test serum caused a greater response than that of the control serum in at least two recipient rabbits when the skin was viewed from the underside.

Statistical analysis

In vivo histamine and methacholine potency values are expressed as the geometric mean±SEM. Values for R_LPC_{50} and $CdynPC_{35}$ were $\log_{10}$ transformed before statistical analysis was performed with the unpaired

Table 1. – Total numbers of cells ($\times 10^4$) and percentage of individual cell types recovered in bronchoalveolar lavage (BAL) fluid from 3 month old HDM-immunized rabbits exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1} from birth

Gas	n	Mononuclear	Neutrophils	Eosinophils	Total cells
Total number of cells $\times 10^4$					
Air	11	168.65 (55.58–335.60)	8.23 (1.43–21.00)	0.00 (0.00–1.23)	169.50 (57.00–339.50)
NO_2	11	135.00 (26.68–424.01)	2.43 (0.00–49.74)	0.00 (0.00–1.30)	140.63 (31.00–473.75)
Percentage of individual cell types					
Air	11	96 (86.0–99.5)	4 (0.5–14.0)	0 (0.0–0.5)	n/a
NO_2	11	96 (22.5–100)	4 (0.0–72.5)	0 (0.0–1.0)	n/a

BAL was performed on the day following the final exposure to either air or NO_2. Data are presented as median value, and ranges in parenthesis. There were no significant differences in cell numbers between groups of rabbits ($p>0.05$). HDM: house dust mite; n/a: not applicable.

t-test. Cell data, PCA titres (given as the reciprocal of the titration end-point) and direct skin test titres (given as the lowest concentration of HDM antigen giving a response) are presented as the median together with the range and were compared using the Mann-Witney test. Results were considered significant at $p<0.05$.

Results

BAL samples

BAL fluid samples were obtained from most animals in the study. Total and differential cell counts recovered from BAL fluid on the day following the last exposure to air or test gas are shown in table 1. Neither the total numbers of cells recovered nor the proportions of cells recovered differed between groups of animals exposed to either 4 ppm NO_2 gas or their corresponding air exposed controls.

Effect of prolonged exposure to NO_2 on airways responsiveness

Figure 1 shows the $R_L PC_{50}$ and $CdynPC_{35}$ values obtained for histamine in littermate groups of HDM-immunized rabbits exposed 2 h·day^{-1}, 5 days·week^{-1} from birth to 3 months of age to either ambient air or 4 ppm NO_2. Figure 2 presents these values obtained for methacholine in the same groups of animals. There were no differences in responsiveness to either histamine or methacholine between HDM-immunized rabbits exposed to NO_2 or their littermate HDM-immunized air-exposed controls ($p>0.05$).

Where possible, full dose-response curves to the bronchoconstrictor agents were completed. No differences in the maximum responses attained to either bronchoconstrictor was apparent, either in R_L or Cdyn measurements, between air exposed and NO_2 exposed HDM-immunized rabbits (table 2).

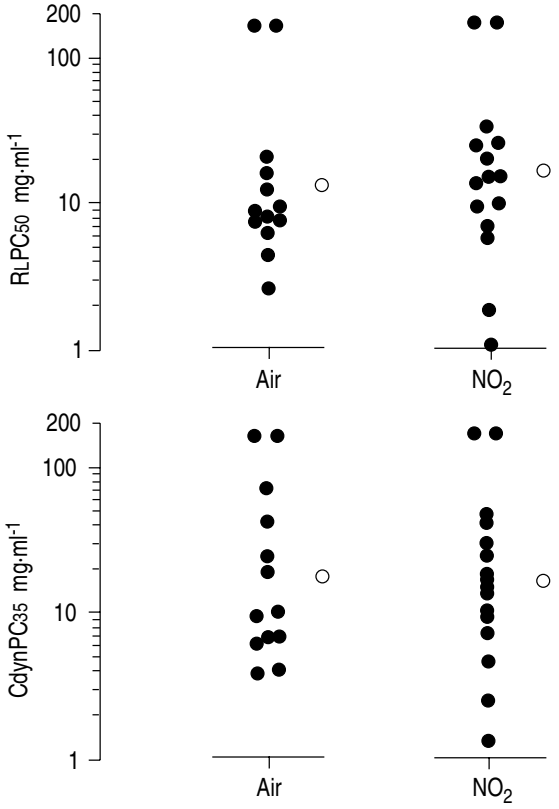

Fig. 1. – Airways responsiveness ($R_L PC_{50}$ and $CdynPC_{35}$) of spontaneously breathing anaesthetized 3 month old HDM-immunized rabbits exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1}, 5 days·week^{-1} from birth to aerosolized histamine. Filled symbols represent values from individual rabbits and open symbols represent the geometric mean±SEM values. $R_L PC_{50}$: provocative concentration of agonist required to elicit a 50% increase in airway resistance; $CdynPC_{35}$: provocative concentration of agonist required to elicit a 25% decrease in dynamic compliance; HDM: house dust mite.

Effect of prolonged exposure to NO_2 on sensitization

Cutaneous responses. Direct skin tests were carried out on 16 rabbits, all of which gave positive immediate skin responses. There was a trend to increased responsiveness in the NO_2 exposed rabbits (fig. 3), which, however,

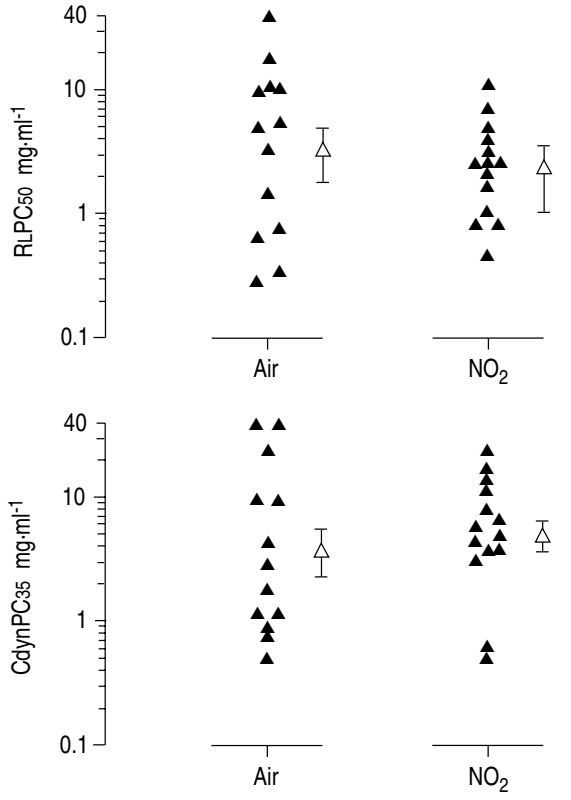

Fig. 2. – Airways responsiveness (RLPC50 and CdynPC35) of spontaneously breathing anaesthetized 3 month old HDM-immunized rabbits exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1}, 5 days·week^{-1} from birth to aerosolized methacholine. Filled symbols represent values from individual rabbits and open symbols represent the geometric mean±SEM values. For abbreviations see legend to figure 1.

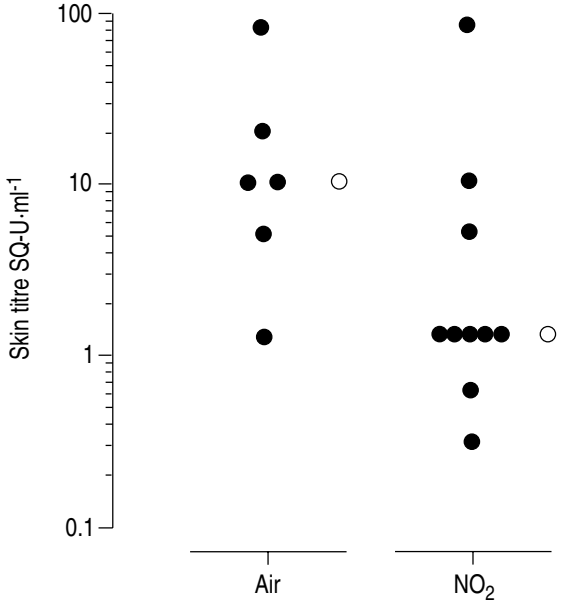

Fig. 3. – Direct skin test titres to HDM antigen in HDM-immunized rabbits exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1} from birth. Serial twofold dilutions of antigen were injected *i.d.* into the shaved dorsal skin of the anaesthetized rabbits. The titration end-point was taken as the lowest amount (SQ-U·site^{-1}) of HDM antigen injected which gave a positive response after 60 min. Filled symbols represent values from individual rabbits and the median values are indicated by the open symbols. HDM: house dust mite.

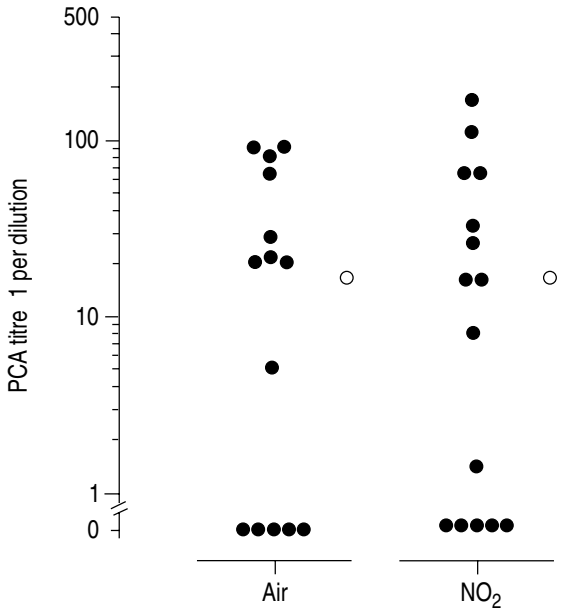

Fig. 4. – PCA titres of sera from HDM-immunized rabbits exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1} from birth. Serial twofold dilutions of sera were injected *i.d.* into the shaved dorsal skin of anaesthetized naive rabbits. After 72 h antigen was administered *i.v.* and the cutaneous responses were assessed after 30 min. The titration end-point was taken as the reciprocal of the highest dilution of serum injected which gave a positive response. Filled symbols represent values from individual sera samples obtained in at least two naive rabbits, and the median values are indicated by the open symbols. PCA: passive cutaneous anaphylaxis; HDM: house dust mite.

was not significant. The median (range) values obtained for skin test titres in air and NO_2 exposed rabbits given as the lowest concentration of HDM antigen (SQ-U·site^{-1}) eliciting a positive response were: air, 10.0 (1.25–80.0), n=6, *versus* NO_2, 1.25 (0.3–80.0), n=10 (p=0.0559).

PCA titres. Serum IgE levels measured by the homologous PCA reaction and used as an index of sensitization are shown in figure 4. In both groups of animals, immunization was not completely successful, with 5 of 14 and 5 of 15 rabbits in air and NO_2 groups, respectively, having PCA titres of zero. The PCA titres did not differ between groups of rabbits exposed to either air or NO_2. The median (range) values obtained for air and NO_2 exposed rabbits given as the reciprocal of the PCA titre were: air, 16 (0–128), n=14, *versus* NO_2, 16 (0–128) n=15 (p>0.05).

Discussion

Our results demonstrate that daily exposure to 4 ppm NO_2 for 2 h·day^{-1}, 5 days·week^{-1} during the first three months of life does not increase airways responsiveness and sensitization in rabbits immunized neonatally by the *i.p.* route to HDM antigen. At the end of 3 months exposure to NO_2 there were no differences in total cell numbers or differential cell counts recovered in BAL fluid samples compared to those obtained from air exposed rabbits. Measurements of basal pulmonary function

Table 2. – Maximal changes of R_L and Cdyn obtained in response to aerosolized histamine or methacholine in 3 month old HDM-immunized rabbits exposed to either ambient air or 4 ppm NO_2 for 2 h·day^{-1} from birth

		Histamine				Methacholine			
Gas	n	$R_L$max $cmH_2O \cdot l^{-1} \cdot s^{-1}$	ΔR_Lmax %	Cdyn,min $ml \cdot cmH_2O^{-1}$	ΔCdyn,max %	$R_L$max $cmH_2O \cdot l^{-1} \cdot s^{-1}$	ΔR_Lmax %	Cdyn,min $ml \cdot cmH_2O^{-1}$	ΔCdyn,max %
Air	13	69.84±7.14	131±18	2.12±0.15	-50±4.7	122.78±16.32	256±55	2.22±0.21	-58±4.6
NO_2	14	73.63±6.85	132±21	1.95±0.17	-58±5.3	162.49±44.52	339±100	1.73±0.14	-61±3.1

Data are presented as mean±SEM. $R_L$max: maximum recorded lung resistance; ΔR_Lmax: maximum percentage increase in recorded R_L; Cdyn,min: minimum recorded dynamic compliance; ΔCdyn,max: maximum percentage decrease in Cdyn; HDM: house dust mite.

revealed no differences in resting values of R_L, Cdyn, maximal Ptp, tidal volume, minute volume or breathing frequency between groups of animals [27]. Neither did values of R_L, Cdyn, maximum Ptp or breathing frequency differ from those of naive animals. Thus, neither immunization nor exposure to NO_2 influences basal pulmonary function parameters.

Airway responsiveness to inhaled histamine and methacholine was measured, and a wide range of values were obtained. However, this is similar to previous findings in man. COCKCROFT *et al.* [28] showed that persistent airways hyperresponsiveness was a characteristic feature of asthma, and described a gradient of increasing airways responsiveness to histamine, from normals to severe asthmatics that is quantitative rather than qualitative, with a considerable degree of overlap. Like human asthmatics, there is a gradient of responsiveness to inhaled histamine from naive animals to rabbits sensitized at birth, with shifts in the measured $R_L PC_{50}$ and $CdynPC_{35}$ [23]. However, no effect of NO_2 exposure was apparent on values obtained for $R_L PC_{50}$ or $CdynPC_{35}$ for either spasmogen. We tested two parameters of allergic sensitization. In direct skin tests, all of the rabbits tested gave an immediate cutaneous response, and although there was a trend for increased responsiveness in the NO_2 exposed rabbits, this did not reach significance. We also used the PCA reaction to obtain indices of IgE levels in sera from all of the animals in the study. Approximately two thirds of serum samples in both groups gave positive PCA results, but the PCA titres did not differ between groups of rabbits exposed to either air or NO_2.

Previous studies have demonstrated increased airways responsiveness to the bronchoconstrictors histamine or methacholine in rabbits immunized within 24 h of birth with the antigens ragweed, horseradish peroxidase or *Alternaria* together with an adjuvant [21–23]. Other studies using the inhaled route for sensitization have used the more common antigen ovalbumin [25, 29]. In the current study, we immunized the rabbits to HDM antigen, since house dust mites are now recognized as important indoor sources of allergens associated with asthma [30].

Our results do not agree with previous observations, that exposure to NO_2 increases allergic sensitization and airways responsiveness in laboratory animals [25, 30–32]. However, other investigators have recently failed to demonstrate any effect of prolonged exposure of rats to NO_2 on the *in vitro* contractile responsiveness of airway smooth muscle [33], or of *in vitro* exposure to NO_2 of bronchial smooth muscle from allergic guinea-pigs [34].

All of the animals used in the present study were immunized by the *i.p.* injection of antigen together with a known adjuvant, $Al(OH)_3$. The lack of influence of these environmental pollutant gases in this model may be related to the *i.p.* route of administration of the antigen, or to the use of the adjuvant $Al(OH)_3$ as a nonspecific stimulant of the immune system. Presumably, during the primary sensitization of asthmatic human subjects the antigen is inhaled into the lungs. It seems probable, therefore, that the route of administration of antigen during the immunization procedure is of importance to the airways responsiveness to bronchoconstrictor agents, such as histamine and methacholine. Indeed, recent reports suggest that 5 ppm SO_2 enhances allergic sensitization in the guinea-pig to inhaled ovalbumin [35, 36].

We are currently using a technique of immunization that allows direct presentation of a particulate antigen to lung tissues in young animals. We hope, in the future, to be able to investigate the putative role of these pollutant gases as adjuvants in sensitization of airway tissues to antigens, such as those of the HDM, and the impact of this on the airways responsiveness to bronchoconstrictor agents.

We conclude that, in rabbits sensitized at birth with *i.p.* HDM antigen and $Al(OH)_3$, daily inhalation of NO_2 at 4 ppm for 2 h·day^{-1}, 5 days·week^{-1} does not affect either the incidence or the degree of sensitization, or influence airways responsiveness to histamine or methacholine.

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