

REVIEW

Exposure of immunologically naive laboratory rodents to antigen via the airways. Where does tolerance stop and sensitization begin?

G. V. Guibas^{1,2}, M. Makris¹, E. Spandou² and K. N. Priftis³

¹Allergy Unit, 2nd Department of dermatology and Venereology, Medical School, "Attikon" General University Hospital, Athens, Greece, ²Laboratory of Experimental Physiology, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece and ³3rd Department of Pediatrics, Medical School, "Attikon" General University Hospital, Athens, Greece

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Summary

Conventional rodent models of respiratory allergy that employ intraperitoneal sensitization to aeroallergen plus adjuvant, have offered greatly to our current knowledge of the pathophysiology of allergic airway diseases. Notwithstanding this significant contribution, non-adjuvant aided sensitization via respiratory presentation of the allergen, is more naturally relevant and more closely mimics the human exposure. Nevertheless, in the experimental setting, primary respiratory exposure to inert antigen is likely to lead to inhalation tolerance. Inasmuch as divergent and discrepant results are often reported in experimental models employing this method of sensitization, we set out to review the relative literature and identify and discuss factors that are liable to interfere in such protocols and modify the immune response, hence leading to variable outcomes. Protocol design features (including the use of anaesthesia, the nature and dosage of the antigen and the strain/age/sex and handling of the animals) as well as environmental factors (including airborne substances, viruses and lipopolysaccharide) have been identified as key modulators of the immune response that evolves, following primary airway exposure of laboratory rodents to aeroallergen. Delineation of the effect of those factors to induction or abrogation of inhalation tolerance can have important implications in the design of both improved experimental protocols of respiratory allergy and methods to intercept sensitization to inert aeroallergens in the clinical field.

Keywords aeroallergen, asthma, experimental protocol, laboratory rodents, sensitization

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Correspondence:

George V. Guibas, Allergy Unit, 2nd Department of dermatology and Venereology, Medical School, "Attikon" General University Hospital, Athens, Greece, Rimini 1, Xaidari, 12462, Greece.

E-mail: george.guibas@gmail.com

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Introduction

Rodent models of allergic airway diseases have contributed a great deal to our current knowledge of the pathophysiology of allergy. Nevertheless, such models are hampered by issues, such as incompatibilities between the human and rodent pathophysiology [1] and the –unnatural– intraperitoneal (i.p) sensitization to antigen coupled with adjuvant [2, 3]. Inasmuch as adjuvants direct the process of Th isotype switching and selectively stimulate IgE responses, their exclusion, alongside with airway administration of antigen, would help design a more naturally relevant protocol [4–6]. Conversely, a substantial body of evidence suggests that non-adjuvant-aided respiratory exposure of naive rodents to inert antigen, is likely to result not in sensitization, but in suppression of IgE responses

and the phenomenon known as inhalation tolerance [7–9].

A change in paradigm has recently been proposed in which, allergic sensitization is related to failing tolerance mechanisms instead of a Th₁/Th₂ imbalance [10, 11]. With this hypothesis in mind, speculation arises that the outcome of respiratory exposure to antigen is determined by factors that can interfere with the normal course of tolerance induction and lead to its ablation.

Thus, main scope of this review is the presentation and discussion of factors that could modulate tolerogenesis following airway exposure of non-sensitized laboratory rodents to antigen. Identification of such factors could lead to the improvement of existing rodent protocols and, by extrapolation to humans, could shed more light to the mechanisms of allergic

sensitization and the proposed paradigm of failing tolerance. Furthermore, as a high proportion of both passively inhaled and intranasally administered antigen is rapidly transported to the gut [12], oral tolerance could also operate in such protocols [13]. Hence, the effect of those factors –or lack thereof– to oral tolerance will also be discussed.

Literature sources

Studies investigating the allergic response following respiratory exposure of naive rodents are scarce, as opposed to those employing the conventional model of airway challenges subsequent to i.p sensitization [14]. Therefore, we set out to find research articles that report both successful sensitization and/or tolerance induction after the implementation of such protocols. Original Research studies that were published in English between 1980 and 2010 were selected through a computer-assisted literature search (Medline). Combinations of relevant key words were used (i.e. airways, sensitization, etc) and the reference lists of the retrieved articles were examined to locate relevant articles that did not show up in the original search. Inclusion criteria for research articles reporting sensitization were lack of adjuvant and strictly respiratory exposure during the whole duration of the protocol, whereas for those articles reporting tolerance was no administration of any substance by any route prior to/during the first respiratory exposure to antigen. The difference in inclusion criteria reflected the necessity for sensitization to be strictly induced by adjuvant-free respiratory exposure, whereas in tolerance studies, i.p administration is often employed following respiratory exposure, to verify potential exposure-induced modulation of antigen-specific IgE.

Immune-regulating factors

A plethora of factors have been identified as key regulators of the immune response subsequent to non-adjuvant-aided primary respiratory exposure of laboratory rodents to aeroallergen (Fig. 1). These include protocol design (antigen, dosage and anaesthesia) with a special focus on the animals used (strain, age, sex and handling) as well as environmental factors (airborne pollutants, active pathogens and lipopolysaccharide -LPS- from various sources).

Protocol design

Antigen

An important part of our current knowledge has originated from rodent models using Ovalbumin (OVA) as a surrogate allergen [3]. Nevertheless, OVA is not clinically

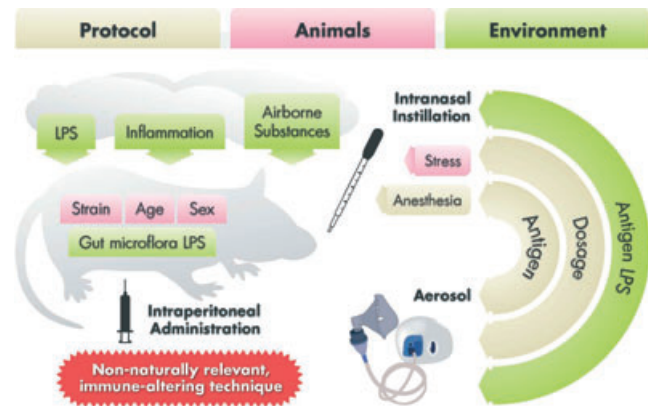


Fig. 1. Factors that could affect the outcome of the immune response following primary intranasal or aerosol exposure of laboratory rodents to aeroallergen. Antigen nature, dosage and LPS contaminants interfere in both aerosol and intranasal techniques, whereas anaesthesia and –immobilization induced and drowning feeling related– stress in the later alone. All other factors are likely to affect sensitization via the airways, regardless of the technique employed. Colours indicate the general group to which each factor belongs. ecru = protocol, pink = animals, green = environment.

cally relevant and the need for adjuvant is partly attributed to this antigens nature, as airway exposure is thought to lead to inhalation tolerance [3, 10, 15]. Notwithstanding this notion, reports of successful sensitization to inhaled OVA are found in the literature (Table 1) [2, 5, 16–21], but are considerably fewer than reports of tolerance induction, some of which are displayed on Table 2 [6, 7, 14, 22–27]. On the contrary, another antigen widely employed in the experimental field, House Dust Mite (HDM) extract, is considered to be extremely pervasive [15]. Indeed, intranasal (i.n) administration of HDM but not OVA, caused marked lung inflammation and Th₂ polarization in a murine model of asthma [26]. Sensitization of mice to –otherwise tolerogenic– inhaled OVA after co-administration with HDM has also been demonstrated [20].

The HDM and OVA are biochemically different entities [26]. OVA preparations consist of a pure 42 kDa protein containing a main immunodominant peptide, OVA_{323–339} [27, 28] and are essentially devoid of additional components [15]. In sharp contrast, HDM extract contains a diverse array of functionally different allergenic proteins including whole mites, nymphs, fecal pellets, eggs and spent culture media [15]. Indeed, mites produce approximately 3000 proteins, 5% of which are considered to be allergenic and HDM allergens are assigned to 21 different groups [15]. The species *Dermatophagoides pteronyssinus* (Der p), is one of the major sources of mite allergen and the groups Der p 1, 3, 6 and 9 are cysteine and/or serine proteases [29], hence characterized by intrinsic enzymatic activity which can create a sensitization-favouring environment

Table 1. Original research papers reporting development of respiratory allergic disease, following ovalbumin (OVA) challenge of rodents that were sensitized through non-adjuvant aided airway exposure

Study	Animals	Sensitization	Challenge	OVA specific IgE	OVA specific IgG	Nasal/Pulmonary inflammation	Cytokines
Renz et al. [5]	Mice BALB/c 8–12 w.o Female	Aerosol	Aerosol	+	Increased after challenge but not after sensitization.	Absence of all types of inflammatory cells	n/r
Hamelman et al. [16]	Mice BALB/c 8–12 w.o Female	Aerosol	Aerosol	+	IgG ₁ +	Increased eosinophils. All other inflammatory cells stable	IL-5+
Herrick et al. [17]	Mice C57BL/6 8–10 w.o Female	Nasal instillation (methoxyflurane anaesthesia)	Nasal instillation (methoxyflurane anaesthesia)	– Total IgE+	IgG ₁ +	+	IL-4+ IL-5+ IL-13+
McCusker et al. [18]	Mice BALB/c 6–8 w.o Male	Nasal instillation (awake)	Nasal instillation (awake)	+	+	+	IL-4– IL-5+ IFN- γ –
Farraj et al. [2]	Mice A/J 6 w.o Female	Nasal instillation (halothane anaesthesia)	Nasal instillation (anaesthesia n/r)	+	n/r	+	IL-4+ (mRNA) IL-5+ IL-13+
Shinagawa and Kojima [19]	Mice A/J, BALB/c, C57BL/6, C3H/HeJ 5 w.o Male	Nasal instillation (enflurane anaesthesia)	Nasal instillation (enflurane anaesthesia)	– (Total IgE increased at various time-points for all strains)	n/r	A/J+ BALB/c – C57BL/6 – C3H/HeJ –	IL-4+ in A/J and BALB/c. IL-5+ in A/J and C3H/HeJ. IL-13+ in A/J
Fattouh et al. [20]	Mice BALB/c 8–12 w.o Female	Nasal instillation of HDM (isoflurane anaesthesia) and subsequent aerosol exposure of OVA	Aerosol exposure of OVA	Increased OVA-specific IgE In HDM/OVA, but not in OVA treated mice	Increased OVA-specific IgG ₁ in HDM/OVA, but not in OVA treated mice	Present in HDM/OVA, but not in OVA treated mice	Increased IL-4, IL-5 and IL-13 In HDM/OVA, but not in OVA treated mice
Li et al. [21]	Mice BALB/c 6–8 w.o Female	Nasal instillation (anaesthesia n/r)	Aerosol	+	IgG ₁ +	Increased eosinophils	IL-4, IL-5 and IL-13 unaffected

+, statistically significant increase; –, no statistically significant alterations; n/r, not reported; w.o., weeks old.

Table 2. Original research papers reporting induction of inhalation tolerance, subsequent to non-adjuvant aided airway exposure of immunologically naïve laboratory rodents to ovalbumin (OVA)

Study	Animals	Sensitization	Challenge	OVA specific IgE	OVA specific IgG	Cytokines
Sedgwick et al. [7]	Rats WAG LOU/M BN 8–12 w.o Male & female	Aerosol	Not applicable	WAG— Lou/M— BN —	WAG — Lou/M+ BN+	n/r
Holt et al. [6]	Rats WAG LOU/M BN Mice BALB/c SJL	Aerosol	Not applicable	WAG rats and SJL mice tolerized with smaller dose than BN rats and Balb/c mice	n/r	n/r
Temelkovsky et al. [22]	Mice BALB/c 8–12 w.o Female	Aerosol	Aerosol	Intraperitoneally Boosted + Non-boosted —	n/r	n/r
Seymour et al. [14]	Mice BALB/c 6–8 w.o Female	Aerosol	Aerosol	—	IgG ₁ + IgG _{2a} +	IL-4— IL-5— IL-10— IFN-γ—
Tsitoura et al. [23]	Mice BALB/c	Nasal instillation (<i>Methoxyflurane anaesthesia</i>)	Not applicable	—	IgG ₁ + IgG _{2a} +	IL-10 and TGF-β uninvolved i n tolerance induction IL-10+
Akbari et al. [24]	Mice BALB/c C57BL/6	Nasal instillation (<i>Methoxyflurane anaesthesia</i>)	Not applicable	n/r	n/r	IL-10+
Swirsky et al. [25]	Mice BALB/c 6–8 w.o Female	Aerosol	Aerosol	—	IgG ₁ +	IL-4— IL-5— IL-13—
Johnson et al. [26]	Mice BALB/c 6–8 w.o Female	Nasal instillation (<i>anaesthesia n/r</i>)	Nasal instillation (<i>anaesthesia n/r</i>)	n/r Total IgE +	n/r	IL-4— IL-5— IL-13— IFN-γ—
Barbey et al. [27]	Mice BALB/c 6–8 w.o Female	Nasal instillation (<i>Halothane anaesthesia</i>)	Not applicable	—	IgG ₁ —	IL-10+ IFN-γ+

+, statistically significant increase; —, no statistically significant alterations; n/r, not reported; w.o., weeks old.

[3, 10, 26]. Indeed, most clinically relevant antigens are strongly associated with protease activity and can confer allergenic potential to innocuous allergens [30]. Such a proteolytic-related adjuvant action, is likely exerted through disruption of cell monolayers, degradation of epithelial tight junctions [20], cleavage of CD25 and CD23 [31, 32], modulation of the balance between IL-4 and IFN-γ [33] and stimulation of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) release [3]. Recently, the involvement of HDM proteases

in anaphylatoxin C5aReceptor-related tolerance mechanisms has also been suggested [34, 35]. Additional mechanisms that could enhance HDM extract allergenicity may include the chitinase activity of Der p 15 and Der p 18 and the robust IgE-binding capabilities of Der p 10 and Der p 11 [15]. Albeit devoid of enzymatic activity, Der p 2, Der p 5 and Der p 7 are also important allergens [29].

In addition to the immunogenic proteins, non-protein components of HDM extract may contribute to its

inherent allergenicity. Although the non-protein content is not well defined, ubiquitously present lipoteichoic acid and peptidoglycans which exert a profound influence on innate immunity are likely contaminants [15]. An ample LPS component is also well documented [15].

Thus far, a substantial body of evidence suggests that even slight changes in the nature of antigen, largely affect the outcome of the immune response [27, 36]. Along these lines, the use of different peptide analogues of OVA_{323–339} lead to variable peptide-dependent immunomodulatory effects in a murine model of asthma [37]. Furthermore, the allergenicity of HDM extract is likely to vary as a result of different production methods and its sheer amount of components [15]. Indeed, inhalation tolerance is often reported following administration of Der p 1 alone [38, 39]. The actual relevance of the HDM-induced inflammatory response to allergic sensitization has also recently been questioned [40].

The plethora of functionally variable components of HDM extract forms the basis of its allergenicity, as opposed to the tolerogenic inclination of the single-protein OVA preparations [15]. Nevertheless, this antigenic diversity constitutes one of HDM major drawbacks, as it hampers standardization and intraprotocol consistency. Conversely, the major drawback of OVA is its innocuous nature and subsequent difficulty to induce sensitization. Thus, the choice between these antigens should be made with consideration to their discrete traits and should be governed by the particular goals of each protocol and the processes that need to be modelled.

Dosage

Inhalation tolerance is probably modulated by both antigen dose and efficiency of uptake, with variable doses likely inducing different dendritic cell-related mechanisms [8, 14, 41]. Such dose dependency has been demonstrated in a study by Seymour *et al.* [14], where aerosol exposure to a wide range of OVA concentrations lead to a dose-dependent induction of OVA-specific IgG and inhibition of IgE responses. Recently, Pauluhn *et al.* [42] have focused on the sensitizing capacity of inhaled polymeric diphenylmethane-diisocyanate (MDI) in rats, in relation to antigen dose, concentration and duration of the exposure. The investigators reported that a high-concentration, short-duration (10 min $\times$ 5 days) sensitization protocol produced a significant inflammatory response following antigen re-challenge, as compared with a low-concentration, long duration (360 min $\times$ 5 days) protocol. They therefore concluded that sensitization to MDI in their experimental model, appears to be dependent on both immunological (dose-dependent) and neurogenic (concentration-dependent) components.

Thus far, a body of evidence is accumulating in support of the notion that tolerance induction is favoured by high-level antigen exposure [7, 14]. Nevertheless, the report by Pauluhn *et al.* [42] suggests that alongside concentration, duration of exposure could be of relevance. Furthermore, in another study, i.n administration of a wide range of OVA doses (0.1–1000 mg) failed to significantly alter the immune response, as evidenced by stable antibody isotype pattern and percentage of eosinophils in BAL fluid [17]. Even though these conflicting reports do not allow for firm conclusions to be drawn, it is likely that low-level antigen exposure can more effectively lead to sensitization. In support of this notion, induction of oral tolerance requires high doses of antigen, possibly due to differences in the gut-lung immune microenvironment [23]. Furthermore, in oral tolerance the dose of antigen fed is the decisive factor over which mechanism takes over [43]. Clonal anergy and deletion are believed to operate in so called high-dose tolerance [44–46] (> 0.5 mg/g in mice /5–20 mg OVA in a single administration [45, 47]), whereas active suppression is responsible for low-dose tolerance [45, 46] (approximately < 0.1 mg/g in mice /maximum 1 mg OVA [45, 47]). Nevertheless, both absence of anergy/deletion [48] and presence of active suppression [45] have been reported in high-dose tolerance, suggesting that all mechanisms could be involved in different degrees in both high and low dose oral tolerance [43].

Anaesthesia

Intranasal application following anaesthesia has been used routinely to immunize rodents because it is inexpensive and not particularly laborious [49]. Nevertheless, anaesthesia probably affects the lung antigen deposition by changing the breathing pattern and airway reflexes of the animal [49, 50]. Furthermore, anaesthesia likely exerts significant immune altering effects *per se*, including alterations on lymphocyte numbers, modulation of T cell-secreted cytokines, increase of corticosterone/ adrenocorticotrophic hormone levels, as well as decrease of T-cell ratios, DNA synthesis and antigen presentation [51, 52].

Although no clear anaesthesia-induced modulation of the sensitization of lab rodents to inhaled antigen is reported in the literature, the capacity of inhaled anaesthetics to exert immune-altering function has been demonstrated [51–62]. The anaesthetics halothane, isoflurane and methoxyflurane are routinely used for i.n rodent challenges [51, 52]. Halothane is extensively metabolized, and its metabolites last up to 12 days [51, 53]. Because of its direct effect on distal airway smooth muscle [54] and depression of vagally mediated airway reflexes [55], halothane causes robust bronchodilation of the small airways [56]. Therefore, intranasal

challenge of halothane-anaesthetized rodents, could lead to increased deposition of allergen in the lung periphery [56]. The intensity of this effect varies depending on anaesthetic concentration, airway caliber and animal species [54]. The mechanisms underlying it may include halothane-induced G-proteins dissociation, increase in protein phosphatase activity, inhibition of m3 muscarinic signalling [54] and production of bronchodilatory prostanoids [57]. Other halothane-induced, immune altering mechanisms could include increased IL-5 mRNA production, as demonstrated for halothane-exposed pre-sensitized C57BL/6 mice in comparison with isoflurane-exposed controls, following i.n OVA administration [56]. Puig et al. [58] reported a halothane-induced enhancement of the immune response of CBI mice to Sheep Blood Red Cells (SBRCs), as evidenced by an increase of IgG-secreting spleen cells in comparison with non-anaesthetized controls. Elena et al. [51], also reported an enhancement of the anti-SRBC response in halothane exposed CBI mice, which could originate from a halothane-driven Th₂ shift. Other, occasionally conflicting, halothane-induced and immune-related effects have been reported in rodents, including increased [59] or decreased [60] numbers and activity of phagocytic cells, suppressed T cell function [60], alteration of cytokine balance [61] as well as reduction of major splenic lymphocyte subsets [51] and splenic IgM and IgG-secreting spleen cells [58].

Isoflurane shares the halothane mechanism, but displays lower bio-transformation rate and bronchodilative capacity [51, 58]. Suppression of pro-inflammatory cytokine production by exposure to isoflurane, consistent with impaired activation of the transcription factor Nuclear Factor- κ B, has been reported [62].

Therefore, it appears that anaesthetic biotransformation and the composition of the vehicle gas mixtures, might affect the homeostasis of the immune system [53]. In this context, ether-anaesthesia at the time of initial i.n exposure of Wistar rats to OVA, was shown in pilot studies in our laboratory to culminate in a significant asthmatic response following antigen re-exposure, as opposed to non-anaesthetized, OVA sensitized and challenged controls [unpublished observations]. Inasmuch as halothane immunomodulatory effects are reported to be both quantitative and qualitative in nature [51] and the use of isoflurane has been suggested to be inappropriate in animal models of acute inflammation [62], anaesthetic treatment could possibly interfere in a specific immune response and divergently affect its fate [51]. On this basis, we propose that different anaesthetic regimens could account in part for the discrepancies in the results of non-adjuvant aided respiratory allergy protocols. Inasmuch as research on the effect of inhaled anaesthesia to the immune response of lab

rodents is scant, we suggest that delineation of the pathways through which different anaesthetics can modulate sensitization to aeroallergens, is a research goal worth pursuing.

Animals

Strain

Genetic factors appear to considerably affect inhalation tolerance induction [63]. Conversely, oral tolerance has been suggested to be extremely potent and with no clear linkages to IgE responder status, as most mouse strains are readily tolerized by a single 20-mg dose of fed OVA [64–66]. Nevertheless, the Major Histocompatibility Complex (MHC) haplotype and strain-dependent differential antigen clearance from the circulation have been reported to interfere in oral tolerance induction [64, 66], thus suggesting a genetic dependency.

In the respiratory system, Sedgwick et al. [7] report that tolerance induction in high-IgE-responder Brown Norway (BN) rats required 10³-fold greater levels of OVA aerosol exposure than the low-IgE-responders Wistar Albino Glaxo (WAG) rats. Similar findings have been reported with high and low IgE responder BALB/c and SJL mice respectively [6]. Okano et al. [4] postulate that the induction of both antigen-specific IgE and nasal eosinophilia by intranasal sensitization with *Schistosoma Mansoni* Egg Antigen (SEA), is strain-dependent with BALB/c, CBA/J and C57BL/6 mice being high, moderate and low responders respectively. Conversely, more robust response of C57BL/6 mice in comparison with the B6C3F1 and BALB/c strain has been reported in a toluene diisocyanate exposure model of asthma [67], suggesting an antigen-dependency of genetically modulated tolerogenesis. Indeed, Fox et al. [68] tolerized all strains of mice of his study to nebulized OVA and suggested that they cannot all be genetically resistant, a conclusion implying the involvement of other factors to genetic control of inhaled tolerance, most likely antigen nature and dosage. Sustained inflammation in A/J but not in BALB/c, C57BL/6 and C3H/HeJ mice following repeated i.n OVA instillations [19] and an increase –albeit transient– in the levels of OVA-specific IgE following aerosol exposure of BALB/c but not C57BL/6 mice have also been reported [69]. Other groups have also reported a genetic dependency of tolerogenesis to inhaled antigens [9, 23, 70, 71].

Therefore, a substantial body of evidence suggests that the fate of the immune response following primary respiratory exposure to allergen is subject to genetic control, which is nevertheless dependent on antigen nature and especially dosage. Indeed, even though a propensity to tolerogenesis is usually associated with low IgE-responder phenotype [63], i.n exposure to the major

Der p 1 T-cell epitope has lead to inhalation tolerance in high Der p 1-responders C57BL/6J mice [38]. Therefore, the immune outcome cannot be genetically predefined irrespective of antigen-related regulating factors.

Age

In the clinical setting, Infancy is a period of great risk of sensitization to environmental antigens [72] as tolerance induction appears to be favoured in mature individuals [72, 73]. Nevertheless, in the experimental field, reports of the sensitizing capacity of discrete aeroallergens in relation to the immune maturity of lab rodents are conflicting, as a propensity to both abrogation [74] and induction [75] of tolerance has been correlated with young age.

A circumvention of inhaled tolerance in OVA-exposed newborn (2 week old) BN rats is reported by Holt *et al.* [74] following subsequent i.p. challenge with OVA. On the contrary, weanling (3 week old), and adult (6 week old) rats demonstrated antigen specific tolerance, as evidenced by a reduced IgE response. Thus, Holt *et al.* proposed that a vital to inhalation tolerance immune component, is not mature at birth, and attributed this to local factors associated with antigen penetration/processing and possibly T-cell immaturity. Along these lines, in pilot studies in our laboratory, we have recently noted a substantial inflammatory response following i.n OVA challenge of Wistar rats that were primary exposed to antigen during weaning (3 week-old) in comparison with rats primary exposed as adults (6 and 8 week-old) [unpublished observations]. Conversely, Al-Garawi *et al.* [75] recently reported in a non-adjuvant-aided asthma protocol, that adult (8 week-old) HDM-sensitized BALB/c mice demonstrated a robust inflammatory response upon antigen re-exposure, as opposed to infants (2 week old). The investigators report immune unresponsiveness of infant mice to HDM, as evidenced by minimal Th₂ cytokine responses and eosinophil recruitment following re-exposure. This age-dependency may be related to elevated levels of TGF-beta and IL-10 in the infant lung in the steady-state and/or an increase in the numbers of inflammatory and CD8a⁺ plasmacytoid dendritic cells in the adult lung.

The presented evidence argues that immune maturity at the time of primary exposure to aeroallergen is indeed involved in tolerance regulation but also that the mechanism in which age interferes is dependent on additional factors, including animal strain and more importantly, antigen nature and lung microenvironment. Along these lines, Phipps *et al.* [76] demonstrated that intranasal exposure to LPS^{low} OVA lead to tolerance in both neonatal (1 day old) and weanling (4 week old) BALB/c mice, whereas the concomitant administration of viral and bacterial-associated molecu-

lar patterns lead to a significant immune response only in neonatal mice.

Therefore, in the experimental field, an age-dependency of sensitization to aeroallergen presented via the airways likely exists, but it is modulated by multiple factors and it relies on a mechanism whose complexity prevents from drawing firm conclusions. Accordingly, induction of oral tolerance is likely regulated by age [64], but in a not well-elucidated manner. Indeed oral tolerance induction in both young (4 week old) and adult (12 week old) rats, albeit via different pathways, has been demonstrated [77]. Further research that takes in consideration strain differences, antigen nature and context of exposure is warranted before age-dependent mechanisms can be delineated.

Sex

Yamatomo *et al.* [78] report an adjuvant-free murine model of allergic rhinitis (AR), where sensitization of male and female CBA/J and BALB/c mice took place via repeated intranasal administration of Phospholipase A₂ (PLA2) and SEA respectively. From the PLA2-sensitized groups, female CBA/J mice produced significantly higher levels of antigen-specific IgE and higher – although not statistically significant-levels of antigen specific IgG₁ than male mice. Nevertheless, in the SEA sensitized groups both male and female BALB/c mice produced similar levels of antigen-specific IgE and IgG₁. Yamatomo *et al.* [78] proposed that male sex hormones, whose receptors are found on thymocytes and bone-marrow cells and are probably related to altered B-cell development, are negatively involved in the production of antigen-specific IgE in the CBA/J-PLA2 model.

Inasmuch as sex-related differences were not seen in the BALB/c-SEA model, even though an identical protocol was employed, the researchers concluded that a sex difference in the initiation of AR does exist in their model, but is likely dependent on both the nature of allergen and the mouse strain.

Thus it appears that sex of the rodents used in airway sensitization protocols has the potential to influence the outcome of the immune response, but most likely in relevance with other regulating factors including animal strain and antigen nature.

Stress

Although no clear stress dependence of the sensitization of lab rodents to antigen via respiratory exposure is reported in the literature, the effect of stress on immune function is too important to go unmentioned in the present review. In fact, taking into consideration the handling of the rodents required for the systemic sensitization (immobilization, traumatic i.p. administration)

as opposed to that required for inhalation exposure, it is reasonable to suspect that the excess stress imposed on the animals exerts immune-altering function [79]. Stress in lab rodents has been reported to cause skin mast cell degranulation [80], enhancement of antigen-induced airway inflammation and hyper-reactivity [81, 82], induction of regulatory T-cell (Treg) production [83], alteration of the Th₁/Th₂ balance [84] and increase of dendritic cell migration and maturation [85, 86]. Those effects are likely mediated through stress-induced modification of the production of Corticotropin Releasing Hormone (CRH) and Substance P [82, 87, 88]. A CRH-dependent, stress-induced increase of antigen uptake in the gut and subsequent abrogation of oral tolerance has also been reported [89]. This effect was duration-dependent, with chronic stress resulting in longer lasting sensitivity to sensitization and was likely mediated through disruption of the epithelial barrier and increase of epithelial permeability [89]. Furthermore, acute stress experienced during primary immunization has been shown to exert adjuvant-like immune effects, increase activated effector Th cells and induce the production of the Th₁ cytokines IL-2 and IFN- γ at the site of antigen re-exposure, hence enhancing cell-mediated immunity (CMI) [90]. Nevertheless, suppressive effects of stress on CMI have also been reported [91], indicating that stress exerts a multi-directional immune effect including both immuno-enhancement and immuno-suppression [92]. Indeed stress has been associated with both a Th₁ and a Th₂ oriented profile [84] depending on factors that include duration of the stressor [79], timing of its application, gender [85], strain [79], age, route of administration and nature of the antigen [85]. Stress in young age (unrelated to the sensitization/challenge period) [93], or even maternal prenatal stress [94], are also reported to alter Th₁/Th₂ differentiation and the response to later airway antigenic challenge.

Thus, it is within reason to propose that for a naturally relevant rodent respiratory allergy protocol to be carried out, an effort to avoid all kind of stressful handling should be undertaken. Therefore, the inhaled route of sensitization is likely preferable to the conventional i.p. paradigm.

Environmental factors

Airborne agents

Environmental factors that can influence the functional outcome of the immune response to inhaled antigens, operate either at the level of respiratory mucosa/regional lymph nodes or systemically [6]. Co-exposure with such agents can promote IgE production, converting what would normally be a tolerogenic signal into a

strong immunogenic stimulus [13, 64]. These agents include free proteases which are abundant in the environment [30] and chemical irritants such as nitrogen dioxide (NO₂) [6] which is present in vehicle exhaust fumes and can be derived from fuel powered engines and household appliances. Indeed, pre-exposure of laboratory rodents to NO₂ prior to antigen inhalation abrogates tolerance induction [6], as is true for exposure to ambient particulate matter derived from fossil fuel combustion and vehicular traffic [21]. Furthermore, tobacco smoke has been demonstrated to facilitate sensitization to OVA in a murine model of asthma, possibly through enhancement of MHC-II gene expression, up-regulation of CD80 (B7-1) and/or production of inflammatory cytokines [69]. Sensitization of laboratory rodents to otherwise tolerogenic aeroallergens has also been reported following exposure to sulphur dioxide (SO₂) released through industrial activity [95], to fossil fuel-combustion-derived ozone [96] and to formaldehyde gas emitted via burning of fuel and formaldehyde-based resins as well as via off-gassing from formaldehyde-containing construction materials and household products [97, 98].

A substantial body of evidence is therefore accumulating in the experimental setting, supporting a strong correlation between such airborne agents and abrogation of inhalation tolerance. Such an adjuvant effect could be facilitated by an increase of bronchial epithelial mucous cells, which has been reported following exposure of lab rodents to environmental pollutants [99]. Furthermore, GM-CSF, a sensitization-favouring cytokine which is secreted by airway epithelial/endothelial cells and lung macrophages [100], is up-regulated after exposure to air pollutants [101]. The ability of environmental toxins to amplify reactive oxidative species (ROS) generation, has also been put forward as a potential mechanism linking environment and atopy [80].

Absolute control of the exposure of lab rodents to potentially immune-altering airborne substances may not be feasible, as even healthy pathogen-free animals housed under strict barrier conditions, are possibly exposed to measurable amounts of such agents [63]. Nevertheless, Inasmuch as the context of the primary encounter to aeroallergen is an important regulator of the ensuing immune response [20, 63, 102, 103], an effort to minimize exposure to airborne pollutants should be undertaken.

Inflammation

Pre-treatment of animals with inflammatory adjuvants could modulate the tolerogenic activity of inhaled antigen [6], as inflammation is considered to be a primary risk factor for hypersensitivity reactions [104]. Inflammatory stimuli at the time of initial respiratory

exposure to an innocuous antigen could promote T cell priming and circumvent suppression [23, 63]. Similarly, inflammation of the gut mucosa is known to alter intestinal permeability, disrupt the cytokine milieu and hinder oral tolerance induction in mice [64].

Viral lower respiratory tract infections are reported to facilitate allergic sensitization to inhaled allergens [105]. Such an adjuvant effect of influenza virus has been reported by several investigators [75, 106–111]. The evidence regarding the involvement of the timing of exposure is conflicting [111, 112]. The mechanism behind it is reported to relate to altered maturation and/or increased airway accumulation of high MHC-II expressing dendritic cells [107] as well as to elevated levels of Toll-Like-Receptor (TLR) 2, 4, 7 and 9, natural killer cells, monocytes, B cells and Th₂ cytokines [75].

Respiratory syncytial virus (RSV) infection concurrent to OVA and ragweed airway exposure has also been reported to facilitate sensitization of mice, in settings that would otherwise lead to tolerance [113–115]. Such an effect could be mediated by virus-induced differentiation of IL-5-producing CD8⁺ T cells [105], by an increase in the permeability of the airway mucosa [116], by GM-CSF up-regulation or by augmented IL-13 airway production [117]. Exposure of BALB/c mice to pneumonia virus of mice, also leads to a circumvention of inhaled tolerance, possibly through up-regulation of lung IL-25 production [118]. An age-dependent abrogation of tolerance through modulation of TLR-7 receptor signalling by viral single-strand-RNA administration, has also been demonstrated [76]. Similar to viral exposure, bacterial antigens, e.g. *Moraxella catarrhalis*, have been reported to exert a DC-related adjuvant effect on airway sensitization [72]. Accordingly, in the gastrointestinal tract, *Escherichia coli* enterotoxin exerts strong adjuvant action [64] and Pertussis toxin is known to be a potent stimulant of IL-4 production [89]. Significantly enhanced mucosal and systemic immune responses have also been reported after intranasal co-administration of any antigen with cholera toxin [119], which is known to have an adjuvant effect both on the respiratory and gastrointestinal mucosa [64, 120]. Indeed, feeding cholera toxin with an unrelated antigen abrogates oral tolerance possibly via abnormal antigen absorption through the columnar gut epithelium, disruption of the balance between Th₁ and Th₂ cells in favour of the latter and/or direct effect on B cells [121].

It therefore becomes apparent that inflammatory signals of both viral and bacterial origin, can exert a potent adjuvant effect and lead to abrogation of inhalation tolerance. Furthermore, exposure to bacterial agents may not only facilitate sensitization to an otherwise innocuous antigen, but can also enhance the immune response to an immunogenic antigen, as is true for concomitant exposure of mice to *Escherichia coli* endotoxin and

cockroach allergen [122]. The substantial experimental evidence pointing to an immune-enhancing capacity of these agents supports the suggestion that strict control of the rodents environment for active pathogens is of utmost importance for carrying out a consistent non-adjuvant-aided respiratory allergy protocol.

Lipopolysaccharide

Endotoxins are ubiquitous airborne LPS compounds derived from Gram-negative bacteria and are potent immune activators [123, 124]. Respiratory exposure of rodents to endotoxin causes airway alterations, including neutrophil infiltration, epithelial cytotoxicity, mucous cell hyperplasia and production of several inflammatory mediators [125]. Nevertheless, LPS effect on induction of respiratory allergy is controversial.

An immune enhancing effect of LPS is reported by several authors [126–128] in relation to LPS-induced inhibition of Treg differentiation [126], induction of GM-CSF secretion by airway epithelial cells [127] and up-regulation of IL-10 production [128]. In fact, mice lacking the LPS receptor appeared unable to mount Th₂ responses in the absence of alum [129], as endotoxin likely provides a danger signal required for sensitization [10]. Conversely, other investigators have demonstrated a protective effect of LPS in regard to asthma development [130–133], which could be IL-12 related. Indeed, LPS is known to be a powerful stimulant of IL-12, which preferentially drives Th₁ responses [3]. A positive modulatory action of LPS has also been suggested regarding oral tolerance, as endotoxin is reported to enhance oral tolerance induction to OVA [64] and LPS from conventional Gastrointestinal Tract (GIT) microflora has been implicated in the maturation of mucosal Tregs [6].

The discrepancies between the effects of endotoxin on induction of respiratory allergy could possibly relate to the timing of the exposure [134]. Furthermore, immune status may be of relevance, as an LPS-dependent immune inhibition is mainly demonstrated following exposure of pre-sensitized animals [130–133] as opposed to an immune enhancement following exposure of naïve animals [108, 122]. The decisive factor although, could in fact be the dose of co-administered LPS [120, 129, 132]. Commercially available OVA is reported to contain variable amounts of LPS [11, 45, 76, 78, 108, 135]. Thus, trace amounts of endotoxin could activate myeloid DCs in a TLR4-dependent manner leading to a Th₂ response, whereas higher doses could induce the production of IL-12 and Th₁ polarization [120, 129]. Indeed, both asthma favouring [108, 129] and inhibiting [135] action of OVA-contaminating endotoxin has been reported. Inhalation tolerance independent of LPS contamination of the OVA aerosol has also been demonstrated [136].

Thus, it appears that the concentration of endotoxin -or lack thereof- in the OVA preparation could affect the outcome of the immune response. Along these lines, the well-documented LPS content of HDM extract is likely to exert adjuvant action [15]. Furthermore, the levels of airborne LPS among laboratories and LPS from GIT microflora are likely to vary. Indeed, even slight alterations in the intestinal flora of rodents maintained under different conditions, could have immune-altering consequences [6]. Inasmuch as thorough scan of the full spectrum of ubiquitous environmental LPS and routine assessment of the minor differences in the GIT microflora cannot be easily incorporated in protocol procedures, control of these factors would be an extremely burdensome task. On the contrary, routine assessment of the LPS-contamination of the employed antigen-especially in the case of OVA-, will likely assist in explaining some of the reported discrepancies in airways sensitization protocols. In any case, reconciliation of such conflicting evidence could derive from the acknowledgement that endotoxin exposure that may elude attention, could divergently direct the immune reaction favouring either sensitization or tolerance.

Concluding remarks

In the present review, we presented research articles pertinent to sensitization of laboratory rodents to anti-

gen via the airways and we discussed the factors that are likely to interfere in such protocols and hence lead to divergent and discrepant results.

Notwithstanding the immense contribution of conventional rodent models of respiratory allergy to our current knowledge, experimental protocols excluding adjuvant and i.p administration of aeroallergens, would be more relevant to the human condition. The pitfalls in creating such protocols are apparent, inasmuch as the outcome of the immune response to inhaled antigen varies widely depending on both protocol design features (such as animals, antigen and use of anaesthesia) and environmental factors that can elude attention (such as exposure to LPS, pollutants or viruses). As the type of immune response that evolves following primary exposure to a certain aeroallergen likely depends on the context in which exposure occurs, identification of those immune-altering factors is of pivotal importance for reliable result evaluation. Furthermore, delineation of the underlying pathophysiology could allow for the design of a more clinically relevant experimental model of allergic airway disease and could have important clinical implications through the development of methods to intercept sensitization to inert aeroallergens.

Conflict of interest

The authors declare no conflict of interest.

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