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Original Article

Comparative studies on nebulizers for antigen inhalation in experimental asthma

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ABSTRACT

The majority of cases of allergic asthma in experimental animals have been provoked by inhalation with mist antigen. Because of the inherent inability of some species to breathe through the mouth, including guinea pigs, mice and rats, an upper airway response may be substantially involved in experimental asthma induced by antigen inhalation. With this in mind, we evaluated three types of nebulizers (a hand-made glassware pressure nebulizer (GPN), a DeVilbiss nebulizer (DN) and an ultrasonic nebulizer (UN)) with respect to their ability to adequately sensitize/challenge guinea pigs by antigen inhalation in bronchial asthma. Several solutions and suspensions, including antigen and $Al(OH)_3$, were atomized with these three nebulizers. The results obtained were as follows: irrespective of the type of solution or suspension used, of the three nebulizers, GPN generated mists with the smallest diameter. The rank order of median diameter of the mists generated was GPN (1.6–2.1 μm) < DN (2.2–3.5 μm) < UN (4.3–4.7 μm). When Evans blue mists produced by the GPN were presented to guinea pigs, 79% of the mist trapped in the whole airway was found in the lung, while 49 and 79% of mists produced by DN and UN, respectively, were already deposited in the upper airway. These results strongly suggest that GPN is more useful for inhalation challenge in experimental asthma in animals.

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INTRODUCTION

Several experimental models have been developed for the study of asthma, particularly the late and early asthmatic responses (LAR and EAR, respectively).[1–10] Guinea pigs have long been preferentially used as good animal models of asthma because their airway smooth muscle responsiveness to many biologically active compounds is very similar to that seen in humans.[11,12] In addition, guinea pigs have been shown to exhibit both EAR and LAR after antigen challenge.[4–6,13–15] In animal models with a dual asthmatic response, guinea pigs are sensitized and challenged with aerosolized antigen. However, guinea pigs breathe through the nose and never through the mouth. Therefore, inhaled mists may be trapped in the upper airway to some degree, consequently suggesting that nasal airway resistance is involved in airway dysfunction, which was thought to have been due to a decrease of lung compliance or an increase of airway resistance in response to the inhalation challenge. Indeed, it has been reported that the LAR in guinea pigs[16] as well as the EAR in rats[17] are composed of both upper and lower airway responses. These reports suggest that in experimental asthmatic animal models, the antigen–antibody reaction should be induced in the lower airway as much as possible.

In contrast, aluminum hydroxide gels ($Al(OH)_3$) have been frequently used as an adjuvant for IgE production by intraperitoneal injection with antigen in animals.[15,18,19] We aimed to sensitize guinea pigs by repetitive exposure of the animals to mist of antigen adsorbed onto $Al(OH)_3$, taking into account its adjuvant properties. However, there are no reports to date on whether the adjuvant gel

suspension is able to be effectively aerosolized and whether the generated mist is sufficiently adsorbed in the airway of guinea pigs.

In the present study we evaluated three kinds of nebulizers, including a hand-made glassware pressure nebulizer (GPN), a DeVilbiss nebulizer (DN) and an ultrasonic nebulizer (UN), for adequate antigen and $Al(OH)_3$/antigen inhalation to sensitize/challenge.

METHODS

Animals

Male 3-week-old Hartley guinea pigs, weighing 250–300 g, were purchased from Japan SLC (Hamamatsu, Japan).

Reagents

Reagents and their sources were as follows: ovalbumin (OA) and NaCl (Wako Pure Chemicals, Osaka, Japan); Evans blue dye (Merck, Darmastadt, Germany); heparin sodium (Takeda Chemical Industries, Osaka, Japan); and sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA). The other reagents used were the highest grade of commercial products available.

Preparation of aluminum hydroxide gel

The $Al(OH)_3$ was prepared by instilling 0.5 mol/L NaOH (100 L) to 0.5 mol/L $Al_2(SO_4)_3$ (100 L) with vigorous stirring; these concentrations were lower than those reported by Levine *et al.*[18] The $Al(OH)_3$ was washed three times with water purified by double distillation (Barnstead, Dubuque, IA, USA) and the Milli Q system (Millipore, Bedford, MA, USA). The average diameter of the gel particles was 0.43 μm when measured by a laser diffraction particle size analyzer (SALD-2000A; Shimadzu, Kyoto, Japan) for the distribution of particle size. The gel was divided and stored at a concentration of 60 mg/mL physiological saline in sealed bottles (300 mL/bottle) at 4°C until use.

Preparation of OA + $Al(OH)_3$ and Evans blue + $Al(OH)_3$

One volume of 2.4 mg/mL OA was instilled into 2 volumes of 60 mg/mL $Al(OH)_3$ suspension under stirring to make a concentration of 800 μg OA/40 mg $Al(OH)_3$ per mL [OA + $Al(OH)_3$]. Evans blue + $Al(OH)_3$ was prepared in the same manner as OA + $Al(OH)_3$ at a concentration of 5 mg Evans blue/40 mg $Al(OH)_3$ per mL. At these concentrations, OA and Evans blue were completely adsorbed onto the gel. Solutions of 5 mg Evans blue/mL (Evans blue) in saline, 16 mg OA/mL (OA) in saline and saline were also prepared.

Mist generation with the nebulizers and inhalation of the mists in the guinea pig

Hand-made glassware pressure nebulizer

As illustrated in Fig. 1, the solution or suspension (approximately 50 mL) was placed in a 2 L transparent flask made of polyvinyl chloride, in which the head of the nebulizer was set through a plug, and was supplied to the nebulizer and circulated with a peristaltic pump (MP-3; Eyela, Tokyo, Japan) at a flow rate of 10 mL/min under stirring. The pressurized air was also supplied to the nebulizer under 101 kPa and 11.4 L air/min. The mists were first introduced into a polypropylene box (1.6 L volume) for the elimination of relatively large particle mists and then guinea pigs were forced to inhale the mists under pentobarbital anesthesia (30 mg/kg, i.p.).

DeVilbiss nebulizer

Following placement of the solution or suspension in the bottom of the DN (Pulmo Neb; DeVilbiss, Somerset, PA, USA), air pressurized by an air pump (Pulmo Aide; DeVilbiss) was supplied to the nebulizer at a flow rate of 7.9 L air/min. Anesthetized guinea pigs were set in the two-chambered box, comprising a head and body compartment, and were forced to inhale the generated mists.

Ultrasonic nebulizer

Antigen, Evans blue or vehicle solution mists produced by the UN (NE-U12; Omron, Tokyo, Japan) were introduced to the head compartment of the two-chambered box, in which the anesthetized guinea pig was placed, with aeration at a rate of 2.0 L/min.

In order to determine the airway distribution of the mists produced by the different nebulizers, Evans blue dye was used as a marker in place of the antigen. Guinea pigs were exposed to mists of either Evans blue + $Al(OH)_3$ or Evans blue alone generated by the three nebulizers at 100 μg Evans blue/animal; the concentration of Evans blue dye was calculated on the basis of respective mist concentrations produced by the

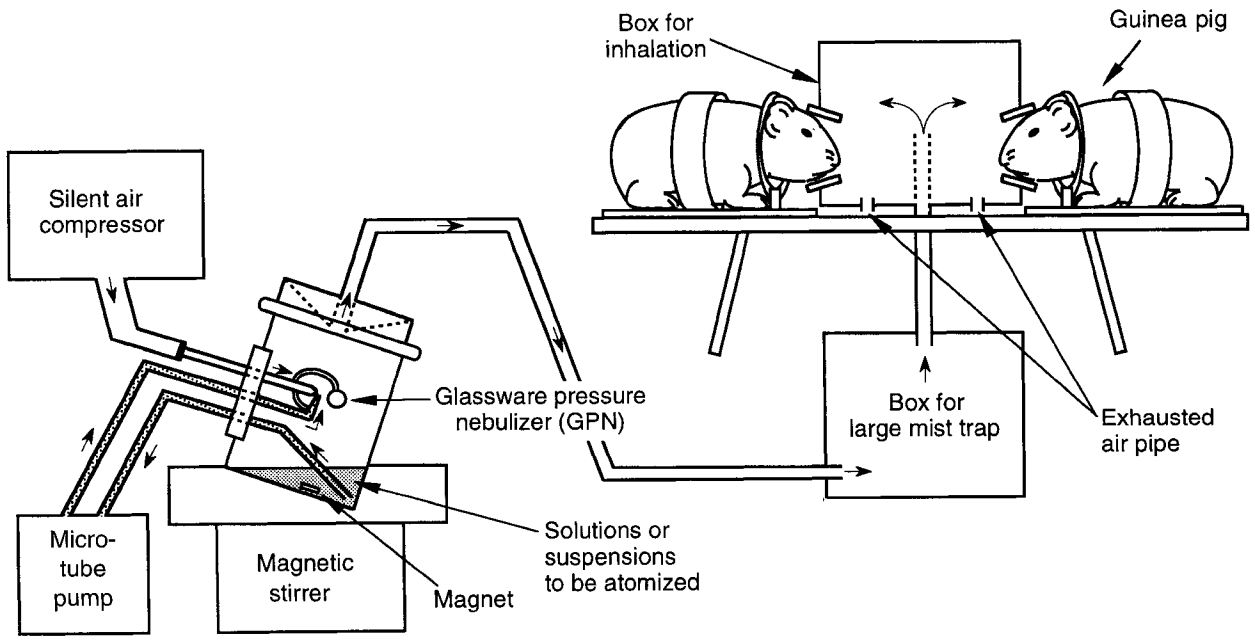

Fig. 1 Schematic illustration of the experimental apparatus for the inhalation of antigen mists generated with a hand-made glassware pressure nebulizer (GPN) in the guinea pig. The conditions for generating mists were air flow rate 11.4 L air/min (at 101 kPa) and micro tube pump flow rate 10 mL/min.

nebilizers and the respiratory volume of the animal for 1 min, which was measured by a two-chambered, double-flow plethysmograph system (Pulmos-I; MIPS, Osaka, Japan).

Measurement of mist particle diameter

The respective mass median diameters of the various mists produced with the three nebulizers were measured by a laser diffraction size analyzer (LDSA-1400A; Tohnichi Computer Applications, Tokyo, Japan).

Measurement of mist concentration

For measurement of mist concentrations in air of Evans blue + $Al(OH)_3$ or Evans blue alone produced by the nebulizers, 400 mL (40 mL × 10 times) of air was collected, at the same position where guinea pigs inhaled the mists, in a 50 mL syringe that had been filled with 10 mL of a mixture of acetone and 0.6 mol/L phosphoric acid (13 : 5): After collection of 40 mL mist air and vigorous shaking, the air was expelled. This manner was repeated 10 times. The dye dissolved in the mixture was colorimetrically measured at 620 nm for estimation of mist concentration.

Determination of the amount and the distribution of mists trapped in the airway

After inhalation of the mist, animals were killed under pentobarbital anesthesia (40 mg/kg, i.v.) and the upper (nasal cavities/pharynges) and the lower (tracheas/main bronchi and lungs) airway tissues were removed. The trapped dye in the airways was measured as described above following extraction according to the method of Katayama *et al.*[20]

RESULTS

Diameter of mists of various suspensions and solutions

Table 1 shows the median diameters of mists generated from the various solutions and suspensions using the three different nebulizers (GPN, DN and UN). Irrespective of the presence of $Al(OH)_3$, GPN produced small mists with median particle diameters of 1.6–2.1 μm. The DN generated a 1.3- to 1.8-fold larger mist with a median particle diameter of 2.2–3.5 μm. In contrast, mists of solutions produced by the UN had the largest diameters (4.3–4.7 μm). The UN is theoretically unable to atomize the $Al(OH)_3$ suspension. In actual fact, it could not

generate a mist containing $Al(OH)_3$ even when a much lower concentration of the gel suspension (5 mg $Al(OH)_3$/mL) was used. From the results obtained with the GPN and DN, it is likely that the concentration or the viscosity of solutions can affect the diameter of the mists formed.

Mist concentration in the air of Evans blue + $Al(OH)_3$ and Evans blue

The concentration of the Evans blue + $Al(OH)_3$ mist generated by the GPN was estimated at 37 μg Evans blue or 300 μg $Al(OH)_3$/L air, which was almost the same as that of Evans blue in terms of the dye concentration, indicating that the GPN produced a mist containing the same ratio of $Al(OH)_3/H_2O$ as that to be atomized. Furthermore, Evans blue + $Al(OH)_3$ was atomized at a constant concentration by the GPN for at least 3 h continuous operation (data not shown). Although the DN could also atomize the $Al(OH)_3$ gel suspension, the mist concentration was only approximately 60% of that of GPN. In addition to this and in contrast to the GPN, DN more easily nebulized the solution rather than the $Al(OH)_3$ suspension. However, the concentration of Evans blue mist generated with the UN was 10-fold higher than generated with the GPN (Table 2).

Amount and distribution of Evans blue + $Al(OH)_3$ and Evans blue alone in the airways

Table 3 shows the amount of mist trapped in the whole airway and its distribution in the upper and lower airways when guinea pigs were exposed to mists of Evans blue +

Table 1. Median diameter of mists of various solutions and suspensions atomized with the hand-made glassware pressure nebulizer (GPN), the DeVilbiss nebulizer (DN) or the ultrasonic nebulizer (UN)

Solution or suspension to be atomized	Median diameter (μm)		
	GPN	DN	UN
Saline	1.6	2.2	4.3
OA	2.1	2.7	4.7
$Al(OH)_3$	2.0	3.1	ND
OA + $Al(OH)_3$	2.0	2.5	ND
Evans blue	1.8	3.1	4.3
Evans blue + $Al(OH)_3$	2.0	3.5	ND

Concentrations of solutions or suspensions are as follows: ovalbumin (OA), 16 mg/mL; $Al(OH)_3$, 40 mg/mL; OA + $Al(OH)_3$, 0.8 mg OA/40 mg $Al(OH)_3$ per mL; Evans blue, 5 mg/mL; Evans blue + $Al(OH)_3$, 5 mg Evans blue/40 mg $Al(OH)_3$ per mL.

ND, not determined.

Table 2. Concentration of mists of $Al(OH)_3$-adsorbed Evans blue (Evans blue + $Al(OH)_3$) or Evans blue atomized with the hand-made glassware pressure nebulizer (GPN), the DeVilbiss nebulizer (DN) and the ultrasonic nebulizer (UN)

Nebulizer	Suspension or solution to be atomized	Concentration of mists (μg Evans blue/L air) (μg $Al(OH)_3$/L air)
GPN	Evans blue + $Al(OH)_3$	37.2 ± 3.8 (297.6 ± 30.4)
	Evans blue	40.8 ± 0.8
DN	Evans blue + $Al(OH)_3$	21.5 ± 5.4 (172.2 ± 42.9)
	Evans blue	71.7 ± 6.1
UN	Evans blue	391.0 ± 41.0

Evans blue + $Al(OH)_3$ suspension or Evans blue solution to be atomized was 5 mg Evans blue/40 mg $Al(OH)_3$ per mL or 5 mg/mL, respectively. Each value represents the mean ± SE of three experiments.

Table 3. Distribution of the mists of $Al(OH)_3$-adsorbed Evans blue (Evans blue + $Al(OH)_3$) or Evans blue atomized with the hand-made glassware pressure nebulizer (GPN), the DeVilbiss nebulizer (DN) and the ultrasonic nebulizer (UN) into the upper and lower airways in the guinea pig

Nebulizer	Suspension or solution to be atomized	Amounts of trapped mists in airway (μg Evans blue/animal) (μg $Al(OH)_3$/animal)	% Distribution of mists trapped in the airway		
			Upper airways	Lower airways	
			Nasal cavities and pharynges	Tracheas and main bronchi	Lungs
GPN	Evans blue + $Al(OH)_3$	65.9 ± 3.4 (527.0 ± 27.2)	38.2 ± 1.6	0.4 ± 0.3	61.6 ± 1.2
	Evans blue	37.8 ± 10.0	21.2 ± 5.9	0 ± 0	78.9 ± 5.9
DN	Evans blue + $Al(OH)_3$	84.5 ± 20.7 (675.6 ± 165.3)	67.5 ± 4.5	0 ± 0	32.5 ± 4.5
	Evans blue	71.3 ± 13.1	49.0 ± 8.7	0 ± 0	51.0 ± 8.7
UN	Evans blue	85.3 ± 20.0	79.2 ± 3.6	0 ± 0	20.8 ± 3.6

Guinea pigs inhaled mists of Evans blue + $Al(OH)_3$ and Evans blue at 100 μg Evans blue/800 μg $Al(OH)_3$ per animal and 100 μg Evans blue/animal as calculated doses, respectively, under pentobarbital (30 mg/kg, i.p.) anesthesia. Each value represents the mean ± SEM of 4–6 animals.

$Al(OH)_3$ or Evans blue alone generated by the nebulizers at a dose of 100 μg dye/animal. The Evans blue + $Al(OH)_3$ mist generated by the GPN was deposited in the whole airway by 66% of the expected amount, while in Evans blue mist with the same nebulizer, 38% of that was found. As for the distribution, considerable amounts of the dye were located in the lung: approximately 60% of the total amount trapped for the Evans blue + $Al(OH)_3$ mist and 80% for Evans blue alone. The rest of the mist was found in the upper airway, nasal cavities and pharynges. In the case of mists generated by the DN, mists of both the solution and the suspension were more effectively captured in the whole airway than were the mists produced by the GPN. However, approximately $\geq$ 50% of the mist by the DN was already trapped in the upper airways. Furthermore, Evans blue mists produced by the UN were most effectively deposited throughout the whole airway compared with mists generated by the other two nebulizers, but only 20% of the mists generated by the UN were located in the lung. For all mists produced by all nebulizers, only a minimal amount was located in the trachea and the main bronchi.

Discussion

In experimental models of asthmatic responses induced by the inhalation of an antigen, spontaneously breathing animals are allowed to inhale the antigen through the nasal cavity, unless specific treatment is administered. Therefore, under these conditions, it is considered that increases in airway resistance or decreases of lung compliance following the inhalation of antigen, which have been assumed as indexes of bronchial asthma, may include nasal resistance. In the present study, we quantitatively evaluated the usefulness of three types of nebulizers with regard to mist generation, mist particle diameter and the way in which the mist reaches and is trapped in the lower airway.

Prior to examination, $Al(OH)_3$, prepared from NaOH and $Al_2(SO_4)_3$ solutions by routine methods, was scrutinized to determine whether the suspended gel was effectively atomized with a pressure nebulizer. The results did not come up to our expectations because the $Al(OH)_3$ mist had a tendency to drop for the larger sizes of gel particles. We overcame this problem by preparing fine particles of $Al(OH)_3$ by instilling low concentrations of NaOH into low concentrations of $Al_2(SO_4)_3$ with vigorous stirring.

The rank order diameter of the mist generated by the nebulizers was GPN < DN < UN, irrespective of the presence of $Al(OH)_3$ in the solutions to be atomized, while UN was unable to produce $Al(OH)_3$ mists. When spontaneously breathing guinea pigs were exposed to these mists, the mists had a tendency to be trapped in the upper airway in proportion to the mist particle diameter. With regard to mist deposition in the whole airway, Evans blue + $Al(OH)_3$ and Evans blue mists generated by the GPN were trapped in the whole airway by approximately 65 and 40%, respectively. It could be presumed that mist trapped in the airway was conveyed to the digestive organs by ciliary movement. However, no detectable amount of dye was found in the mouth, esophagus or stomach. Therefore, it could be that a mist with a small particle size inhaled into the airway is exhaled again without coming into contact with the airway mucosal membrane. It can be conceived that relatively large mists generated by the DN and UN were easily trapped by the nasal mucosa and once they were trapped, they induced the further accumulation of newly inhaled mists in the nasal cavity because of a decreasing patency of the upper airway cavity. This suggests that if these two nebulizers are to be used for the induction of experimental asthma by a definite dose of antigen, inhalation with antigen mists at a high concentration over a short period of time provides better conditions for antigen inhalation than do relatively low concentrations for a long period of time.

For generating fine mists at a constant concentration with GPN, at least three points, described below, are important: (i) when air is supplied to the air nozzle of the GPN, a considerable flow rate (11.4 L air/min in the present study) is required under a constant pressure (101 kPa in the present study); (ii) a constant supply rate of solutions or suspensions to be atomized to the air nozzle is necessary; and (iii) a box should be set between the nebulizer and the inhalation box to trap large mists.

Interestingly, as seen for DN and particularly GPN, a considerable difference was noted either in the amount of mist trapped in the whole airway or in the distribution of the mist trapped in the upper and the lower airways between Evans blue + $Al(OH)_3$ and Evans blue mists. As the median diameters of the mists between the suspension and solution were quite similar, these differences cannot be due to mist particle size. It is conceivable that the suspension mist has more of a chance to collide against the airway mucosal membrane because of the higher density of the $Al(OH)_3$ mist than the solution mist.

With regard to the diameter of mist, the mean size of aerosol particles generated with the UN and measured by a particle size measurement system[21] identical to that used in the present study has been reported to be 4.9 μm,[13] which is similar to our present value. As shown in the present experiments, the mist generated with the UN was deposited in the upper airway four-fold more than in the lower airway. Thus, it is strongly suggested that in both EAR and LAR observed in guinea pig asthma models induced by inhalation with UN-generated antigen mists,[13–15] changes of the patency of the upper airway cavity are involved in EAR and LAR. In fact, there are reports that the upper airway contributes to LAR in guinea pigs after inhalation with aerosolized antigens from the nose[16] and that challenge by topical instillation of antigen solution into the nostril comes in two waves (at 10 min and 6 h) of increases of nasal airway resistance.[22] Similarly, recent experiments in our laboratory have indicated that challenge by inhalation with Japanese cedar pollen, more than 99.999% of which was located in the upper airway,[23] induced dual responses of the upper airway (T Nabe *et al.*, unpubl. obs., 1997). Taking these results and the results of the present study into consideration, the efficient distribution of antigen mists to the lower airway is crucial for research into asthma using experimental animals.

Taking into consideration the fact that it is important that sufficient concentrations of antigen mists reach the lower airway at the time of challenge rather than at sensitization, it would seem that location of $Al(OH)_3$ mists high in the upper airway is not an issue because $Al(OH)_3$ may not be used at the time of challenge.

We have examined whether experimental asthma is able to be induced by sensitization/challenge by repeated inhalations with GPN- and UN-generated mists of OA solution at 20 μg/animal, once every 2 weeks, which is a considerably low dose compared with that used in other reports.[4–6,13–15] Consequently, EAR but not LAR emerged in guinea pigs exposed to GPN-generated mists, with increases of serum titers of not only γ_1 but also IgE.[24] Meanwhile, repeated inhalations of the UN-generated mists failed to induce any responses (data not shown). In contrast, when OA + $Al(OH)_3$ GPN-generated mists were inhaled for sensitization, both EAR and LAR were observed.[24] Therefore, it appears that the production of anaphylactic antibodies is preferentially induced by antigen reaching the lower airway, even at relatively low doses, and that $Al(OH)_3$ plays some role in the occurrence of LAR.

In summary, it is concluded that the GPN is preferentially useful for inhalation sensitization and particularly for challenge in experimental animals on the basis of the facts that: (i) the mist produced by the GPN effectively reaches the lung and settles there; and (ii) the GPN can generate a mist of relatively high and constant concentration over several hours.

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