



ASSESSMENT OF CHEMICALS FOR THEIR POTENTIAL TO INDUCE RESPIRATORY ALLERGY IN GUINEA PIGS: A COMPARISON OF DIFFERENT ROUTES OF INDUCTION AND CONFOUNDING EFFECTS DUE TO PULMONARY HYPERREACTIVITY

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Abstract—Guinea pigs were sensitized either to selected low molecular weight chemicals known to induce respiratory allergy in humans, trimellitic anhydride (TMA), toluene diisocyanate (TDI), or diphenylmethane-4,4'-diisocyanate (MDI), or to ovalbumin (OA) as a positive control. In most instances, sensitization was induced either by repeated intradermal injections or by a single brief (15-min) high-concentration inhalation exposure. For TMA the repeated high dose intradermal injection regimen was compared with a single low dose intradermal injection regimen. Additionally, the effectiveness of the single 15-min induction protocol was compared with that of five consecutive inhalation exposures each of 3 hr/day. Animals were challenged 2–3 wk later by exposure to the substance used for induction, either as the free chemical or as a hapten–protein conjugate, and with increasing concentrations of acetylcholine (ACh). Challenge with the parental or conjugated hapten was used to assess compound-specific immediate-onset respiratory hyperreactivity, while ACh challenges were used to identify non-specific airway hyperreactivity. After intradermal sensitization with either MDI or TMA guinea pigs challenged with the corresponding hapten–protein conjugate showed a moderate incidence of immediate-type respiratory responses. However, the highest incidence of unequivocal allergic responses was evident from challenge with the hapten rather than with the protein conjugate, although these responses were only elicited with slightly irritant concentrations. After challenge with irritant concentrations of TDI, animals sensitized intradermally did not experience characteristic changes in respiratory patterns. On challenge with ACh and the TDI–protein conjugate these same animals showed an increased airway hyperresponsiveness although characteristic stereotypic breathing patterns, as observed in sensitized animals challenged with TMA, TMA–protein conjugate, or OA, were not detected. Comparison of the intradermal and inhalation induction regimens indicated that prior encounters with irritant haptens by inhalation reduces the concentration required to elicit airway hyperresponsiveness. This finding supports the conclusion that in animals sensitized and challenged by inhalation, irritant respiratory responses may be misconstrued as immediate-onset allergic responses. It appeared that the low dose single intradermal injection protocol is more effective in sensitizing guinea pigs than the high dose repeated injection protocol.

INTRODUCTION

Experimental and clinical studies have suggested that the aetiological development of an increased airway hyperresponsiveness to asthmogenic chemicals may result from brief high concentration exposures. Increasing awareness of the potential for chemicals to induce allergic respiratory hypersensitivity has resulted in attempts to develop sensitive test models appropriate to evaluate this endpoint (Botham *et al.*, 1988; Griffiths-Johnson and Karol, 1991; Karol *et al.*, 1989; Thorne and Karol, 1988). Currently there exists no well-validated model for the prospective identification of the potential for chemicals to cause respiratory

sensitization. Various modes of hapten or antigen administration and methods for detection of sensitization have been used. Guinea pigs sensitized by inhalation to either a free or a protein-bound chemical have been induced to exhibit changes in respiratory patterns following inhalation challenge with the same chemical, either as the free compound or as the protein conjugate. In the past, practically all such models have relied on the use of the guinea pig, a species that is known to be sensitive to agents inducing bronchoconstriction and in which respiratory function and respiratory hypersensitivity can be measured readily (Campos and Church, 1992). Current evidence suggests that respiratory hypersensitivity would better be classified as immunogenic or neurogenic (Meggs, 1993). The distinction between neurogenic and immunogenic asthma is limited to the inducing chemicals because the two types are indistinguishable following the release of mediators. The two mechanisms might be differentiated by testing guinea pigs with challenge concentrations of specific antigens

Abbreviations: ACh = acetylcholine; API = acetylcholine provocation index; GPSA = guinea pig serum albumin; GSD = geometric standard deviation; MDI = diphenylmethane-4,4'-diisocyanate; MMAD = mass median aerodynamic diameter; OA = ovalbumin; SD = standard deviation; TDI = toluene diisocyanate; TMA = trimellitic anhydride.

and non-allergic, non-specific chemical stimuli while monitoring the respiratory pattern. This situation is complicated further since chemical haptens may evoke non-specific irritant as well as specific allergic hypersensitivity responses and the responses occurring may be clinically indistinguishable.

More recently it has been found that alterations in respiratory parameters can also be provoked in guinea pigs sensitized dermally by inhalation challenge with unconjugated, free chemicals or with hapten-protein conjugates (Botham *et al.*, 1988; Hayes *et al.*, 1992; Pauluhn and Eben, 1991). The current investigations focus on the development of an animal model that permits identification of the asthmogenic potential of low molecular weight chemicals. Specifically, responses resulting from induction by both intradermal and inhalation protocols were compared. This approach was taken to address difficulties with data interpretation that may arise from respiratory tract irritation at challenge that results from previous inhalation sensitization. Non-specific airway hyperresponsiveness was assessed by inhalation challenge with aerosolized acetylcholine (ACh) while compound-specific hyperresponsiveness was identified by challenge with the hapten, either as the free chemical or the protein conjugate.

MATERIALS AND METHODS

Test material. Diphenylmethane-4,4'-diisocyanate (MDI) and toluene diisocyanate (80% 2,4-TDI, 20% 2,6-TDI) are proprietary products of Bayer AG (Leverkusen, Germany). Trimellitic anhydride (TMA) and ovalbumin (OA) (grade V) were obtained from Aldrich Chemicals and Sigma Chemie (Germany), respectively. Conjugates were synthesized using guinea pig serum albumin (GPSA) obtained from Sigma Chemie. The molar ratios were as follows: TMA-GPSA conjugate, 26:1; MDI-GPSA conjugate, 27:1; TDI-GPSA conjugate, 23:1.

Sensitization. Outbred guinea pigs of the Hartley strain from various breeders were used. In most studies a minimum of eight guinea pigs were used for each experimental group. Standard intradermal induction was carried out by repeated injections on days 0, 2 and 4 [injection volumes, 2×50 – $100 \mu\text{l/day}$; concentrations, 5–10% (w/v)]. Additionally, for TMA a modified intradermal induction was used [volume, $100 \mu\text{l}$; 0.3% (w/v), single or triple administration]. Generally, corn oil-acetone (1:4, v/v) served as a vehicle. OA was dissolved in saline (10%, w/v). Inhalation induction was carried out by a single 15-min high concentration exposure (TMA, MDI and TDI), quadruple 15-min exposure (OA), or, for TMA, five consecutive exposures (3 hr/day).

Elicitation of respiratory sensitization. Starting on day 21, after an observation period of approximately 2–3 wk, the guinea pigs were challenged by inhalation with either the hapten or with the hapten-GPSA conjugate. In some studies the animals were given

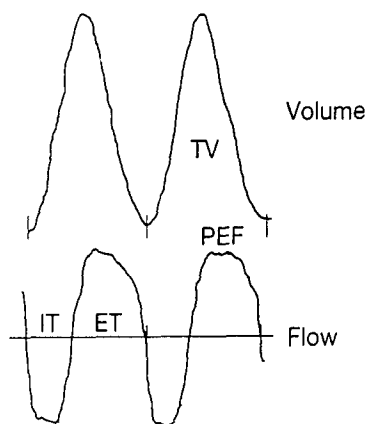


Fig. 1. Analysis of immediate-onset hypersensitivity responses in guinea pigs using nose-only flow-plethysmographs. The flow signal is integrated to obtain the tidal volume. The parameters evaluated were: IT, inspiratory volume (sec); ET, expiratory volume (sec); ET/IT (dimensionless); rate = breathing frequency (breaths/min); tidal volume (ml); MV, respiratory minute volume (ml/min); PEF, peak expiratory flow rate (ml/sec); $\text{PEF} \times (\text{IT} + \text{ET})/\text{TV}$ (dimensionless). Calculations were based on time averages of 10–15 sec.

also an additional ACh challenge by inhalation 1 day after the hapten challenge. Each ACh challenge consisted of exposure to three progressively increasing concentrations of ACh [0.1, 0.3 and 1.0% (w/v)], each for 15 min. For immediate-onset respiratory measurements animals were acclimatized to the plethysmograph and after collection of baseline parameters for approximately 15 min (exposure to air), they were challenged for about 30 min, followed by post-challenge measurements of about 30 to 45 min. Measurements were made with four guinea pigs simultaneously as described previously (Pauluhn and Eben, 1991). The immediate-onset respiratory parameters evaluated are summarized in Fig. 1.

Atmosphere generation and analysis. Atmospheres of MDI were generated using a modification of the condensation aerosol generation system described by Rapaport and Weinstock (1955). TMA dust atmospheres were generated exactly as described previously (Pauluhn and Eben, 1991) and TDI atmospheres were generated by methods described for other volatile isocyanates (Pauluhn and Eben, 1992). Aerosols of aqueous solutions of OA, protein conjugates or ACh were made as described elsewhere (Pauluhn, 1994). TMA and protein concentrations were determined by gravimetric analysis while isocyanates were analysed by published methods (Dunlap *et al.*, 1976). Particle-size distributions of ACh were respirable [mass median aerodynamic diameter (MMAD) = 0.92 – $1.09 \mu\text{m}$, geometric standard deviation (GSD) = 1.29 – 1.37]. Particle-size distributions of TMA and MDI (MMAD/GSD) were about $2.5 \mu\text{m}/2.1$ and 1.2 – $2.0 \mu\text{m}/2.2$ – 2.5 , respectively.

Data recording and evaluation. For each respiratory parameter, individual baseline data were used to calculate the mean $\pm 3 \times$ standard deviation (SD) as

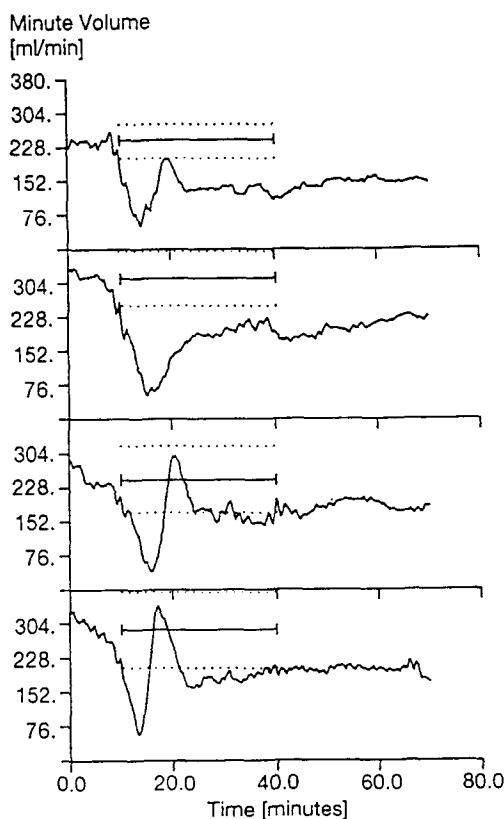


Fig. 2. Breathing patterns from guinea pigs sensitized with trimellitic anhydride (TMA) by repeated intradermal injections and challenged with TMA [collection of baseline values approximately 10 min; duration of challenge 30 min (—) recovery approximately 30 min]. Dotted lines represent $3 \times \text{SD}$ of baseline measurements.

shown in Fig. 2. Responses exceeding the mean $\pm 3 \times \text{SD}$ were considered to be positive. Responses were ranked as follows. The highest rank of response was accorded with increases in the $\text{PEF} \times (\text{ET} + \text{IT})/\text{TV}$ parameter (where PEF = peak expiratory flow, ET = expiratory time, IT = inspiratory time, TV = tidal volume), an intermediate response was a change in the respiratory minute volume, and alterations in respiratory rate and tidal volume were considered to indicate minimally positive responses. A hallmark of allergic hypersensitivity in this model is the waveform breathing pattern (see Fig. 2), since this breathing pattern has never been observed in control animals. The ACh provocation index (API) is a means to identify sensitivity to non-specific bronchoprovocation and was calculated as:

$$\text{API} = 100 \times \left(\sum_{i=c_1}^{c_3} R_i \times f_i \right) / N$$

R_i = number of responders per ACh concentration: $c_1 = 0.1\%$ ($f_1 = 10$), $c_2 = 0.3\%$ ($f_2 = 3$) and $c_3 = 1.0\%$ ($f_3 = 1$), respectively, for the three different concentrations per challenge. N is the number of animals challenged.

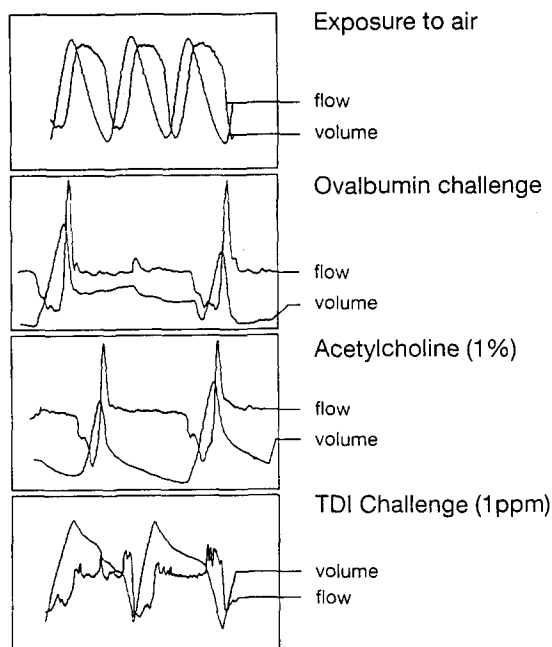


Fig. 3. Breathing patterns indicating an allergic respiratory hypersensitivity response in guinea pigs sensitized, by repeated intradermal injections, to ovalbumin (OA) or TDI. 2 to 3 wk after the final induction with the sensitizing agent the animals were challenged with approximately 12 mg OA/ m^3 followed 1 day later with acetylcholine challenge, or with 1 ppm TDI.

RESULTS AND DISCUSSION

Breathing patterns of guinea pigs sensitized to and challenged with either OA or TDI are presented in Fig. 3. Changes in the flow-volume patterns recorded during OA and ACh challenge were similar and were characterized by an apnoeic period between expiration and inspiration. Guinea pigs challenged with an irritant concentration of TDI (about 1 ppm) also experienced a decreased respiratory rate. However, this change is attributable to a reflexively induced bradypnoeic period between inspiration and expiration, a response reported to be characteristic for upper respiratory tract irritation in rodents. To overcome the limitations caused by TDI-induced respiratory tract irritation, additional challenge exposures were performed with ACh and with the TDI-GPSA conjugate to assess airway hyperresponsiveness to non-specific and specific stimuli. As summarized in Table 1, the API from guinea pigs previously challenged with TDI was approximately three times the value of concurrent controls. On challenge with the TDI-GPSA conjugate seven out of 12 animals (58%) experienced a breathing pattern different from that observed during the hapten challenge (increased respiration rate, decreased TV) which is consistent with the performance of an asthmatic lung.

Guinea pigs were sensitized to MDI either by repeated intradermal injections or by a single brief high-concentration exposure to evaluate whether

Table 1. Summary of immediate-onset responses following sensitization and challenge with MDI, TMA, TDI or OA

Compound	Induction regimen*	Induction concn/ (w/v)	Approximate challenge concn (mg/m ³ air)	Immediate response (%) / anaphylaxis (%)	API†	Conjugate challenge response (%)
MDI	Control	—	12	25/0	63	38
	Control	—	30	13/0	ND	38
	Control	—	60	50/0	100	13
	id	6 × 5%	12	25/0	38	38
	id	6 × 5%	30	88/0	ND	38
	id	6 × 5%	60	88/0	338‡	38
	ih	135 mg/m ³	12	75/0	100	88
TMA	Control	—	36	0/0	ND	0
	id	6 × 5–10%	45	100/0	ND	88
	id	3 × 0.3%	25	100/38	ND	ND
	id	1 × 0.3%	25	88/75	ND	ND
	ih	290 mg/m ³	31	88/0	ND	ND
	5 ih	51–118 mg/m ³	36	25/0	ND	25
TDI§	Control	—	4	44/0	38	9
	id	6 × 0.5%	4	20/0	100	58
	ih	45 mg/m ³	4	88/0	125‡	38
OA	control	—	12	13/0	113	ND
	id	6 × 10%	12	100/13	129	ND
	4 ih	13 mg/m ³	16	88/50	ND	ND

ND = no challenge performed

*id = intradermal; ih = single, 15-min inhalation exposure; 5 ih = five consecutive inhalation exposures, each for 3 hr/day; 4 ih = four consecutive inhalation exposures, each for 15 min/day. Controls were given vehicle during sensitization.

†Acetylcholine provocation index.

‡Two out of eight guinea pigs died during the acetylcholine challenge.

§Conversion factor: 1 ppm (ml/m³) = 7 mg/m³ air.

previous inhalation exposures alter the responsiveness when challenged with slightly irritant concentrations. Animals sensitized intradermally experienced hypersensitivity responses only during challenge exposures with slightly irritant concentrations of MDI (about 30 mg/m³). However, animals challenged with approximately 60 mg MDI/m³ showed a conspicuous increase in non-specific airway hyperresponsiveness during the ACh provocation (API = 338; two out of eight animals died). Challenge with the MDI-GPSA conjugate did not increase the sensitivity of this test model (most responses observed were weak or equivocal). A comparison of responses elicited after intradermal sensitization and after sensitization by a single 15-min exposure to 135 mg MDI/m³ is shown in Table 1. During MDI challenge (about 12 mg/m³) hypersensitivity responses were observed in 75% of the animals, whereas from intradermally sensitized animals this challenge concentration was too low to elicit obvious breathing pattern changes.

The intradermal sensitization protocol also appeared to be predictive for the strong respiratory allergens TMA and OA. One of the hallmarks of allergic respiratory hypersensitivity, the waveform breathing pattern (Fig. 2), was observed during challenge exposures with OA, TMA, or TMA-GPSA, but not following challenge with MDI, MDI-GPSA, or TDI-GPSA. Following repeated high-dose intradermal administrations of either TMA or OA a high incidence of immediate-onset responses was observed (Table 1). This evidence suggests that a quenching of immune response or a desensitization of animals as a result of the high doses administered did not occur.

However, comparison of immediate-onset response data from animals sensitized to TMA by the high-dose repeated intradermal regimen [5–10% (w/v)] and the low-dose single or triple injection regimen [0.3% (w/v)] revealed marked induction-dependent effects (Table 1). Although the incidence of responses occurring during challenge with TMA was by and large the same, the magnitude of the responses that occurred after challenge was considerably more pronounced following the low-dose injection regimen (most guinea pigs on the low-dose injection regimen died in anaphylactic shock).

Also the single, high-concentration inhalation induction regimen appeared to be predictive. However, as was apparent from results obtained during the ACh bronchoprovocation challenge, the non-specific airway hyperreactivity was increased. Apparently, induction exposures to high concentrations of chemical irritants may lead to desquamation of the airway epithelium and hence removal of the barrier to chemical irritants, so that chemical irritants may reach and trigger the irritant receptor at lower concentrations. As demonstrated with TMA dust, the repeated exposure (5 × 3 hr/day for 5 consecutive days) to such high concentrations may result in tissue destruction and accordingly in diminution of response.

Conclusions

These studies demonstrate that elicitation of respiratory hypersensitivity is challenge-concentration dependent and that the concentrations used should

slightly exceed the threshold concentration for irritation to maximize the incidence of response. Sensitization by inhalation increases the susceptibility to irritant stimuli and thus confounds the selection of the most appropriate concentration for challenge. The combined approach of evaluating several breathing parameters with both hapten (free or conjugated) and ACh challenge provides a promising method of evaluating specific hypersensitivity responses. It is critically important to assess the respiratory irritancy of the compound under investigation. For potent irritant substances such as volatile isocyanates, challenge with the hapten-protein conjugate minimizes the likelihood of confounding specific hypersensitivity responses with those merely of irritation. It also appears that the intradermal-induction inhalation-challenge protocol is less susceptible to influences related to irritation-induced airway hyperreactivity than the inhalation-induction inhalation-challenge protocol. Moreover, as it is consistent with the conclusions of Hayes *et al.* (1992), the sensitivity of this intradermal induction animal model is increased when a low-dose rather than a high-dose induction regimen is used.

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