ELSEVIER

Toxicology 96 (1995) 37–50

TOXICOLOGY

A two-centre study for the evaluation and validation of an animal model for the assessment of the potential of small molecular weight chemicals to cause respiratory allergy

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Received 27 May 1993; accepted 15 November 1994

Abstract

This study evaluated a single intradermal injection model in the guinea pig with subsequent inhalation challenge and serological analysis as a method to predict the potential of chemicals to induce respiratory allergy. Four known respiratory allergens (trimellitic anhydride, diphenyl methane diisocyanate, phthalic anhydride and toluene diisocyanate (TDI)) were screened by two industrial research laboratories using this protocol. Dinitrochlorobenzene, a potent contact allergen, was included as a negative control material. In both laboratories, the respiratory allergens, but not the contact allergen, induced high titre antigen-specific antibodies in treated animals. The inhalation challenge results were similar in both laboratories but were less conclusive in that exposure to free TDI failed to induce pulmonary responses, probably because it fails to penetrate to the deep lung in sufficient concentration. Although the assay shows promise as a means of identifying chemical respiratory sensitisers, its use as a routine screen for the prediction of the ability of materials to induce respiratory allergy in man is probably questionable.

Keywords: Animal model; Validation; Chemicals; Respiratory allergy; Isocyanate; Anhydride

1. Introduction

It is well recognised that a number of small molecular weight chemicals such as isocyanates, acid anhydrides and platinum salts can cause occupational respiratory disease mediated by specific IgE antibody (Hagmar et al., 1987; Murdoch and Pepys, 1987). These chemicals alone are not allergenic but combine with tissue proteins to form hapten protein conjugates which are capable of both inducing a specific immune response and eliciting an allergic reaction in a previously sensitised individual. The immune response may be directed against the hapten or against the new antigenic determinant created by the formation of the hapten protein conjugate (Patterson et al., 1982a).

The list of chemicals known to cause occupa-

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SSDI 0300-483X(94)03007-0

tional respiratory allergy is expanding as a result of better medical surveillance and diagnostic procedures, and because of the increase in the number of chemicals and chemical processes used in industry. There is therefore a need for a suitable model to predict which chemicals have the potential to cause respiratory allergy before these chemicals are introduced into the working environment.

A number of animal models have been proposed which involve the induction of sensitisation by inhalation, intrabronchial instillation, topical application, intraperitoneal injection or intradermal injection using free chemical or hapten protein conjugates. The degree of sensitisation in the treated animals was assessed by the measurement of specific antibody and airway responsiveness after inhalation exposure to free chemical or hapten protein conjugates (Karol, 1980; Karol et al., 1980, 1981; Chen and Bernstein, 1982; Patterson et al., 1982b; Murdoch and Pepys, 1984; Botham et al., 1988).

More recently, a guinea pig model has been described which involved the induction of sensitisation by a single intradermal injection of free trimellitic anhydride (TMA) (Botham et al., 1989). The degree of sensitisation in treated animals was assessed by the measurement of specific antibody by passive cutaneous anaphylaxis (PCA) and enzyme linked immunosorbent assay (ELISA) analysis, and by the measurement of respiratory rate after nose-only exposure to an atmosphere containing a respirable dust of free TMA. High titre antigen specific antibody was induced using this protocol and TMA was consistently able to elicit pulmonary reactions in treated guinea pigs after nose-only exposure of the sensitised animals. This was in marked contrast to earlier studies in which challenge with atmospheres containing TMA–guinea pig serum albumin conjugate (TMA–GSA) failed to elicit pulmonary reactions in animals sensitised by inhalation exposure to free TMA (Botham et al., 1988).

The purpose of this investigation was to evaluate the suitability of a single intradermal injection model with a subsequent inhalation challenge and serological analysis for use as a routine method to predict the potential of chemicals to induce respiratory allergy. Four recognised occupational respiratory allergens (trimellitic anhydride, diphenyl methane diisocyanate, phthalic anhydride and toluene diisocyanate) (Hagmar et al., 1987; Murdoch and Pepys, 1987) were screened by two industrial research laboratories (Zeneca Central Toxicology Laboratory[1], Macclesfield, Cheshire and Unilever Environmental Safety Laboratory[1], Sharnbrook, Bedford) using this protocol. Specific antibody levels were measured by PCA and ELISA analysis. Dinitrochlorobenzene, which is a potent contact allergen in both man and animals but does not cause respiratory allergy (Botham et al., 1989), was included as a negative control material.

2. Materials and methods

2.1. Animals

Male and female Dunkin–Hartley guinea pigs, with an initial weight range of 250–350 g, were obtained from either Harlan Porcellus Breeding Ltd. (Firgrove Farm, Cross in Hand, Heathfield, Sussex, UK) or Unilever Research (Sharnbrook, Bedford, UK).

2.2. Test materials

Trimellitic anhydride (TMA) and phthalic anhydride (PHA) were supplied by the Aldrich Chemical Co. (Gillingham, Dorset, UK). Toluene diisocyanate (TDI) was supplied by ICI Colours and Fine Chemicals (Blackley, Manchester, UK) as a 80:20 mixture of the 2,4- and 2,6-isomers. Diphenyl methane diisocyanate (MDI) was supplied by ICI Polyurethanes (Blackley, Manchester, UK).

2.3. Control material

Dinitrochlorobenzene (DNCB) was supplied by the Aldrich Chemical Company.

2.4. Additional materials

Dinitrofluorobenzene (DNFB) was supplied by the Aldrich Chemical Company. Guinea pig serum albumin (GSA) was supplied by the Sigma Chemical Company (Poole, Dorset, UK).

[1]Throughout this article * denotes Zeneca Central Toxicology Laboratory, Macclesfield, Cheshire, UK, and ** denotes Unilever Environmental Safety Laboratory, Sharnbrook, Bedford, UK.

2.5. Test procedure

Sensitisation of the guinea pigs was induced by a single intradermal injection of the test or control material (100 μl) on day 1. The control animals received vehicle alone. A blood sample was taken from each of the treated and control animals on day 19 for serological analysis. Three days later on day 22 the animals were challenged by an inhalation exposure to the appropriate test or control material.

2.6. Sensitisation procedure

Test and control materials were suspended or dissolved in a suitable vehicle (corn oil alone, acetone and corn oil, or acetone and physiological saline). The test animals were sensitised by a single intradermal injection of the appropriate test of control material (100 μl) into the scapular region on day 1 of each experiment. For each of the materials the maximum concentration which produced no signs of systemic toxicity and only minimum local irritation was used. The concentration for each of the materials was determined in a preliminary study. The control animals in each experiment received vehicle alone.

2.7. Inhalation challenge procedure

Immunologically sensitised animals respond to an inhalation challenge with the sensitising antigen exhibiting an increase in respiratory rate and a decrease in tidal volume (Karol et al., 1980). This response can progress to a slow, gasping breathing pattern reflecting severe bronchostriction. Animals were placed in individual plethysmograph whole body restraining tubes and placed in the exposure chamber to allow nose only exposure to the test or control material. After a brief settling period, the animals were monitored for 10 min to establish the normal background rate of respiration. This was followed by a 15-min challenge with an atmosphere containing the appropriate test or control material. After exposure the animals were removed from the exposure chamber and monitored for a further 15 min or until their breathing had returned to the normal background rate. The animals were monitored constantly during the pre-challenge, challenge and recovery periods for any signs of respiratory distress. Respiratory rates and patterns were recorded by either optical plestysmography* as described previously (Botham et al., 1988) or pressure plethysmography** using the Respiratory Analysis Programme (Physiologic Ltd., Arkwright Crescent, Newbury, Berkshire, UK).

The pulmonary reactions were categorised for each individual animal based on the changes in respiratory rate as follows:

Severe response: A rapid decrease in respiration rate to 70% or less of the normal background rate within the 15-min challenge period (this response may be preceded by an increase in respiratory rate).

Moderate response: An increase in respiration rate to 130% or more of the normal background rate within the 15-min challenge period.

No effect: Changes in respiration rate within 71–129% of the normal background rate within the 15-min challenge period.

2.8. Atmosphere generation and analysis

TMA and PHA were generated as dusts from freshly milled test material using either a Wrights Dust Feed Mechanism* (Adams, London, UK) supplied with dry compressed air or argon; or a Venturi Dust Generator** (Unilever Research Laboratories) supplied with compressed air. DNBC was generated either as a dust* using a Wrights Dust Feed Mechanism or as an aerosol** generated from a freshly prepared solution of DNBC in ethanol using a Misty-Ox Nebuliser (Medic-Aid Ltd., Hook Lane, Rose green, Bognor Regis, West Sussex, UK). MDI was generated as a condensation aerosol from molten MDI heated to 67°C* or 80°C**. A saturated vapour was generated by directing a jet of dry air onto the surface of the molten MDI which was then cooled to produce a condensation aerosol when passed down a cooling column. TDI vapour was generated by bubbling dry air through TDI at 35°C. The TDI–GSA* conjugate aerosol was generated from a 2% aqueous solution using a concentric glass jet atomiser. The solution was supplied to the generator at a steady flow rate using a peristalic pump.

Atmospheric concentrations of TMA–, MDI–, PHA–, DNCB– and TDI–GSA conjugate were determined gravimetrically using filter sampling and

the particle sizes and distributions were determined using a Marple Cascade Impactor* (Schaefer Instruments, Wantage, Oxon, UK) or using a TSI APS33 Aerodynamic Particle Sizer** (TSI Incorporated, St. Paul, MN). The concentration of TDI vapour was determined either by drawing an atmosphere through sintered glass impingers containing a solution of *N*-4-nitrobenzyl-*N*-*n*-propylamine in toluene to trap any isocyanates present as urea derivatives, followed by quantification by HPLC* (Dunlap et al., 1976), or by using a Miran 980 Infrared Spectrometer** (The Foxboro Co., South Norwalk, CT).

2.9. Preparation of hapten–guinea pig serum albumin conjugates

To enable the detection of hapten specific antibodies, hapen–guinea pig serum albumin (hapten–GSA) conjugates were prepared by allowing free hapten to react with guinea pig serum albumin (GSA) in aqueous solution (a DNFB–GSA conjugate was prepared to detect DNCB specific antibodies and was used because of its greater reactivity with protein) (Botham et al., 1989).

*Laboratory 1**. TMA–, MDI–, PHA–, TDI– and DNFB–guinea pig serum albumin conjugates were prepared by dissolving 200 mg of GSA in 20 ml of borate buffer (0.05 M boric acid, 0.05 M potassium chloride, 0.035 M sodium hydroxide; pH 9.4) and slowly adding 60 mg of the appropriate hapten with mixing. The conjugate preparations were then stirred on ice for 30 min. Each conjugate preparation was dialysed extensively against distilled water. The resulting conjugate preparations were lyophylised and stored below −10°C.

*Laboratory 2***. TMA–GSA conjugate was prepared by dissolving 100 mg of GSA in 5 ml of 9% sodium hydrogen carborate and slowly adding 100 mg of TMA with mixing. The conjugate preparation was incubated at 37°C for 5 min and then cooled to room temperature. Free hapten was separated from the conjugate preparation by gel filtration in phosphate buffered saline (PBS; pH 7.2) using Sephadex G25 (fine, Pharmacia LKB Biotechnology, Milton Keynes, Bucks, UK). The conjugate preparation was concentrated to 20 ml (5 mg protein/ml) using ultrafiltration (Diaflo PM10 filter, Amicon Corp., Denvers, MA 01923, USA). The resulting conjugate preparation was then stored below −10°C.

MDI– and TDI–guinea pig serum albumin conjugates were prepared by dissolving 200 mg of GSA in 10 ml of borate buffer (pH 9.4) and slowly adding 60 mg of the appropriate hapten with mixing. The conjugate preparations were then stirred on ice for 30 min. Free hapten was separated from the conjugate preparations by gel filtration. Each of the conjugate preparations was concentrated to 40 ml (5 mg protein/ml) using ultrafiltration and stored below −10°C.

PHA–GSA conjugate was prepared by dissolving 200 mg of GSA in 10 ml of distilled water. Three 35-mg samples of PHA were added slowly with mixing at hourly intervals. The pH of the mixture was maintained at between 9.0 and 9.6 with the addition of 1 M sodium hydroxide. Free hapten was separated from the conjugate preparation by gel filtration 1 h after the addition of the third sample of PHA. The conjugate preparation was concentrated to 40 ml (5 mg protein/ml) using ultrafiltration and stored below −10°C.

DNFB–GSA conjugate was prepared by dissolving 200 mg of GSA in 10 ml of 1 M sodium hydrogen carbonate and slowly adding 20 mg of DNFB with mixing. The conjugate preparation was incubated at 27°C for 30 min and another 20 mg of DNFB was added slowly with mixing. The conjugate preparation was incubated for 2.5 h at 27°C and 20 mg of DNFB was added slowly with mixing. The conjugate preparation was incubated for 2.5 h at 27°C. Free hapten was separated from the conjugate preparation by dialysis against PBS (pH 7.2). The conjugate preparation was concentrated to 40 ml using ultrafiltration and stored below −10°C.

2.10. Evaluation of the hapten–GSA conjugate preparations

In order to confirm the binding of the haptens (TMA, MDI, PHA, and TDI) to GSA in each of the conjugate preparations, 2,4,6-trinitrobenzene sulphonic acid (TNBS) was added to each of the conjugate preparations and to GSA alone. The TNBS binds to the amino groups on the protein (Snyder and Sobocinski, 1975) and therefore any

reduction in binding for the conjugate preparations when compared with the binding with GSA alone also confirmed the binding of the hapten to the protein. The binding of DNFB to GSA in the conjugate preparation was assessed by comparing the absorbance profile of the conjugate preparation with that of free GSA at 360 nm and 410 nm, an increase in the absorbance indicating binding of DNFB to the protein.

The suitability of each of the conjugate preparations for use in the detection of hapten specific antibodies was also confirmed using ELISA analysis and appropriate positive and negative control guinea pig serum samples. All the conjugate preparations were able to bind to the appropriate specific antibodies.

2.11. Detection of hapten specific antibodies in the guinea pig serum samples

On day 19 of each of the experiments a blood sample was taken from all the animals by cardiac puncture and the serum samples were assayed for the presence of specific IgG1 antibodies using PCA and/or ELISA analysis.

Passive cutaneous anaphylaxis (PCA) analysis. Serum samples taken from test and control animals in each of the experiments were diluted in sterile physiological saline and 0.1-ml aliquots were injected intradermally along the shaved flanks of paired naive recipient guinea pigs. Between 4 and 6 h later the guinea pigs were treated by intravenous* or intracardiac** injection of 1 ml of the challenge solution (containing 2.5 mg/ml of the appropriate conjugate preparation and 0.5%* or 1%** Evans Blue dye). The intradermal injection sites were scored for the size of the blue reaction 20–30 min after the challenge. A skin reaction of ≥ 3 mm $\times$ 3 mm* or ≥ 5 mm $\times$ 5 mm** was considered to be positive.

Enzyme linked immunosorbent assay (ELISA) analysis. Disposable plastic wells Nunc Maxisorp F96* (Nunc, Copenhagen, Denmark) or Removastrip** (Dynatech Labs Ltd., Billingshurst, West Sussex, UK) were coated by an overnight incubation at 4°C* or room temperature** with 100 μl of the appropriate hapten–GSA conjugate preparation (5 μg/ml in carbonate buffer; pH 9.6). The antigen coated wells were then washed in phosphate buffered saline containing 0.05% Tween 20 (PBS/Tween). The antigen coated wells were incubated with 100 μl of the guinea pig serum samples (diluted 1/10 or 1/50 in PBS/Tween and in four-fold dilutions to 1/163 840 or 1/819 200) at 37°C for 30 min* or room temperature for 2 h**. The wells were then incubated with 100 μl of rabbit anti-guinea pig IgG1 (ICN Biologicals, High Wycombe, Bucks., UK) at a dilution of 1/2500* or 1/2000** in PBS/Tween at 37°C for 30 min* or room temperature for 2 h**, followed by peroxidase labelled goat anti-rabbit IgG (ICN Biologicals) at a dilution of 1/2500* or 1/2000** in PBS/Tween at 37°C for 30 min* or room temperature for 2 h**. The wells were washed in PBS/Tween between each step. The wells were finally incubated with 100 μl of substrate solution [*o*-phenylene diamine (Sigma Chemical Co. Ltd.), 0.8 mg/ml in citrate phosphate buffer (pH 5.0) containing 0.02% hydrogen peroxidase] at room temperature for 5 min. The reaction was stopped by the addition of 50 μl of 0.5 M citric acid to each well. The absorbance of the well contents was measured at 450 nm by means of a Multiscan* (Flow Laboratories, Irvine, Scotland, UK) or an MR4000 Microplate Reader** (Dynatech Labs Ltd.). The reaction was considered positive if the absorbance of the serum dilution was greater than 0.1 and greater than twice the optical density value for the control serum sample, included in each assay, at the corresponding dilution. Antibody titres were expressed as the reciprocal of the highest serum dilution giving a positive result.

3. Results

The results are summarised in Table 1.

3.1. Trimellitic anhydride (TMA)

*Laboratory 1**. Five groups of guinea pigs were injected intradermally on day 1 with concentrations of TMA in corn oil ranging from 0.003% to 0.3%. Each group of animals was exposed to the same inhalation challenge concentration of TMA (achieved concentration 7.8–16.8 mg/m^3) on day 22. This experiment was designed to establish the relationship between the sensitising dose, the antibody response and the pulmonary response. A

Table 1
Summary of results

Chemical	Laboratory 2**		Laboratory 1*	
	Pulmonary response	Serological response	Pulmonary response	Serological analysis
TMA	+++	+++	+	+++
MDI	+++	+++	++	+++
PHA	++	+++	+	+++
TDI	–	+++	– (+[b])	+++
DNCB	±[a]	±	±	+

Symbols: +++, positive response in the majority of the treated animals; ++, positive response in a high proportion of the treated animals; +, positive response in a number of the treated animals; ±, positive response in only a small number of the treated animals; –, no positive responses in any of the treated animals.
[a]Similar responses were also observed in the control animals.
[b]Using a TDI–protein conjugate.

summary of the results is given in Table 2. The serum samples from the majority of the animals in each of the treatment groups contained IgG1 TMA-specific antibodies as measured by PCA analysis. There was a sensitisation dose related increase in IgG1 antibody titre as measured by ELISA analysis. All the groups contained some animals which gave a positive pulmonary reaction on inhalation challenge; however, there was no obvious dose response between the pulmonary reactions and the sensitising concentration. In fact, one of the animals in the group sensitised with 0.003% TMA (the lowest sensitisation concentration gave a severe pulmonary reaction which then progressed to anaphylaxis and the death of the animal).

Table 2
Summary of the serological and pulmonary results following sensitisation with increasing concentrations of TMA

Sensitisation dose (%)	Antibody analysis		Pulmonary response category (number of animals in each category)		
	ELISA titre (serum dilution^{-1})	PCA result (no. of animals positive)	No effect	Moderate response	Severe response
*Laboratory 1**					
0 (control)	< 10 (8)	0/8	8	0	0
0.003	< 10 (4) 40 (2) 640 (2)	3/5	5	0	2
0.01	160 (3) 640 (2) 2560 (3)	7/8	6	0	2
0.03	200 (4) 800 (2) 3200 (2)	8/8	4	2	2
0.1	800 (1) 3200 (5) 12 800 (2)	7/8	6	1	1
0.3	12 800 (5) 51 200 (3)	8/8	4	3	1

ELISA: IgG1 antibody titre, dilution^{-1} (number of animals).

*Laboratory 2***. Five groups of guinea pigs were injected intradermally on day 1 with 5% TMA in corn oil. Each group was exposed to a different inhalation challenge concentration of TMA (target concentrations ranging from 5 to 50 mg/m^3) on day 22. This experiment was designed to establish the relationship between the inhalation challenge concentration and the pulmonary response. A summary of the results is given in Table 3. The serum samples from all the animals in the treatment groups contained IgG1 TMA-specific antibodies as measured by PCA analysis. A pool of the serum samples from all the animals in the five test groups gave an IgG1 antibody titre of 12 800 in the ELISA analysis. No TMA-specific antibodies were detected by PCA or ELISA analysis in any of the serum samples taken from the control animals. There was no appreciable difference in the overall numbers of animals giving a positive pulmonary reaction in each of the groups. The inhalation challenge concentrations of 5 mg/m^3 and 10 mg/m^3 produced a similar incidence of positive pulmonary reactions to those in the other three groups but these were predominantly in the moderate response category rather than the severe category.

3.2. Diphenyl methane diisocyanate (MDI)

*Laboratory 1**. In order to establish the sensitisation dose response, groups of guinea pigs were injected intradermally on day 1 with concentrations of MDI in corn oil ranging from 0.0003% to 1%. The animals were exposed to an inhalation challenge concentration of between 18 and 42 mg/m^3 on day 22. A summary of the results is given in Table 4. The serum samples from all the animals in the groups sensitised with 0.01% MDI and above contained IgG1 MDI-specific antibodies as measured by PCA analysis. There was a sensitisation dose related increase in IgG1 antibody titres as measured by ELISA analysis up to a concentration of 0.1%. Above this sensitisation concentration there was no further increase in the antibody titres. Positive pulmonary reactions on inhalation challenge were observed in animals from all the groups sensitised to doses of 0.01% and above. However, there was no substantial increase in the incidence or severity of these pulmonary responses with the increase in the sensitisation dose.

*Laboratory 2***. A group of guinea pigs was injected intradermally on day 1 with 0.1% MDI in 6% acetone in corn oil. The animals were exposed to an inhalation challenge concentration of 55 mg/m^3 MDI on day 22. A summary of the results is given in Table 4. The serum samples from all the animals in the treatment group contained IgG MDI-specific antibodies as measured by PCA analysis and by ELISA analysis, the ELISA antibody titres ranging from 12 800 to 204 800. A pul-

Table 3
Summary of the pulmonary results following sensitisation with 5% TMA

Target inhalation challenge concentration (mg/m^3)	Achieved inhalation challenged concentration (mg/m^3)	Pulmonary response category (number of animals in each category)		
		No effect	Moderate response	Severe response
*Laboratory 2***				
Control group				
50.0	51.0	8	0	0
Treatment groups				
5.0	7.1	2	5	1
10.0	11.2	0	5	3
25.0	22.8	1	1	6
35.0	40.4	0	3	5
50.0	57.2	1	1	6

Table 4
Summary of the serological and pulmonary results following sensitisation with MDI

Sensitisation dose (%)	Antibody analysis		Pulmonary response category (number of animals in each category)		
	ELISA titre (serum dilution^{-1})	PCA result (no. of animals positive)	No effect	Moderate response	Severe response
*Laboratory 1**					
0 (control)	< 10 (16)	0/16	16	0	0
0.0003	< 10 (4) 10 (2) 40 (1) 160 (1)	0/8	6	0	0
0.001	< 10 (2) 40 (1) 160 (1) 640 (3) 2560 (1)	0/8	7	0	0
0.003	160 (1) 640 (2) 2560 (4) 10 240 (1)	2/8	7	0	0
0.01	2560 (4) 10 240 (4) 20 480 (6)	15/15	5	3	7
0.03	20 480 (8)	8/8	6	0	1
0.1	10 240 (8) 51 200 (8)	16/16	9	2	4
0.3	51 200 (8)	7/7	3	1	3
1.0	51 200 (6)	5/5	2	0	4
*Laboratory 2***					
0 (control)	< 50 (8)	0/8	8	0	0
0.1	12 800 (2) 51 200 (9) 204 800 (1)	12/12	2	7	3

ELISA: IgG1 antibody titre, dilution^{-1} (number of animals).

monary reaction was observed in 10 out of 12 of the animals in the treatment group and 3 of these reactions were in the severe response category.

3.3. Pathalic anhydride (PHA)

*Laboratory 2***. In an initial experiment guinea pigs were injected intradermally on day 1 with 0.3% PHA in 6% acetone in corn oil. The animals were exposed to an inhalation challenge concentration of 44 mg/m^3 PHA on day 22. A summary of the results is given in Table 5. The serum samples from all the animals in the treatment group contained IgG1 PHA-specific antibodies as measured by PCA and by ELISA analysis, the ELISA antibody titres ranging from 800 to 51 200. However, there was no pulmonary reaction in any of the treated animals on inhalation challenge.

Investigation of this lack of responsiveness to inhalation challenge revealed that the physicochemical characteristics of the PHA used for the inhalation challenge may have influenced the induction of a pulmonary response. Particulate PHA oxidises very rapidly and therefore it was decided to freshly grind the PHA just prior to its use at challenge, and that very dry air or an inert gas such as argon should be used in the generation process.

The study was repeated using freshly ground

Table 5
Summary of the serological and pulmonary results following sensitisation with PHA

Sensitisation dose (%)	Antibody analysis		Pulmonary response category (number of animals in each category)		
	ELISA titre (serum dilution^{-1})	PCA result (no. of animals positive)	No effect	Moderate response	Severe response
*Laboratory 1**					
Experiment 1 (argon)					
0 (control)	< 10 (8)	0/8	6	1	1
0.03	10 (2)	2/8	6	1	1
	40 (4)				
	640 (2)				
0.1	10 (2)	6/8	5	1	1
	160 (1)				
	640 (4)				
	2560 (1)				
0.3	640 (1)	7/8	4	0	4
	2560 (3)				
	10 240 (4)				
Experiment 2 (dry air)					
0 (control)	< 10 (8)	0/8	8	0	0
0.03	640 (5)	7/7	6	0	1
	2560 (2)				
0.1	2560 (6)	8/8	7	0	1
	10 240 (2)				
0.3	2560 (1)	8/8	5	0	3
	10 240 (7)				
*Laboratory 2***					
Experiment 1					
0 (control)	< 50 (7)	1/8	8	0	0
	200 (1)				
0.3	800 (7)	12/12	12	0	0
	3200 (2)				
	12 800 (2)				
	51 200 (1)				
Experiment 2 (dry air)					
0 (control)	ND	ND	6	0	1
0.3	3200[a]	ND	4	1	7

ELISA: IgG1 antibody titre, dilution^{-1} (number of animals). ND, not determined.
[a]Pool of the serum sample from the animals in the test group.

PHA at an inhalation challenge concentration of 52 mg/m^3 in dry air. In this experiment a pulmonary reaction was observed in 8 out of 12 animals in the treatment group and 7 of these responses were in the severe response category. This confirmed the need for freshly ground PHA to produce a pulmonary response at inhalation challenge. A severe pulmonary response was also observed in 1 out of 8 of the control animals, presumably due to an irritant response to PHA.

*Laboratory 1**. In two subsequent experiments, using this modified inhalation challenge regime, groups of guinea pigs were injected intradermally on day 1 with 0.03%, 0.1% or 0.3% PHA in corn oil. The first experiment used argon as the inhalation challenge generation medium and the animals were exposed to an inhalation challenge concentration of 11–29 mg/m^3 on day 22. The second experiment used dry air as the inhalation challenge generation medium and the animals were exposed

to an inhalation challenge concentration of 9–48 mg/m^3 on day 22. A summary of the results from the two experiments is given in Table 5. The numbers of animals giving a positive result in the ELISA analysis were comparable between the two experiments. However, the IgG1 antibody titre values were slightly higher in each of the three groups in the second experiment and this was reflected in the PCA analysis where a higher proportion of the animals in the second experiment gave a positive result. As with the antibody analysis, the results of the inhalation challenge were comparable between the two experiments. The only clear evidence of a PHA-specific pulmonary response was at the highest sensitisation dose of 0.3%, where 4 out of 8 animals in the first experiment and 3 out of 8 animals in the second experiment exhibited a positive pulmonary reaction and all these reactions were in the severe response category. Some positive responses were observed in the groups sensitised with the lower concentrations of PHA but a similar response was also observed in one of the control groups, again presumably due to an irritant response to PHA.

3.4. Toluene diisocyanate (TDI)

*Laboratory 2***. A group of guinea pigs was injected intradermally on day 1 with 0.1% TDI in 6% acetone in corn oil. The animals were exposed to a nominal challenge concentration of 2 p.p.m. estimated from the bubbler settings used in preliminary studies to achieve this concentration (the actual concentration was not measured because of problems caused by coating of the lens in the analyser) on day 22. A summary of the results is given in Table 6. The serum samples from all the animals in the treatment group contained IgG1 TDI-specific antibodies as measured by PCA and by ELISA analysis, the ELISA antibody titres ranging from 3200 to 51 200. However, there was no pulmonary reaction in any of the animals in the treatment group at inhalation challenge. The use of higher inhalation challenge concentrations in preliminary experiments resulted in a depression in the respiratory rate of both the treated and control animals, which is consistent with an irritation response to the TDI vapour.

*Laboratory 1**. Two groups of guinea pigs were injected intradermally on day 1 with 0.1% TDI in

Table 6
Summary of the serological and pulmonary results following sensitisation with TDI

Sensitisation dose (%)	Antibody analysis		Pulmonary response category (number of animals in each category)		
	ELISA titre (serum dilution^{-1})	PCA result (no. of animals positive)	No effect	Moderate response	Severe response
*Laboratory 1**					
0 (control)	<50 (8)	0/8	8	0	0
0.1	3200 (3)	15/15	15	0	0
	12 800 (8)				
	51 200 (1)				
	>51 200 (3)				
*Laboratory 1** *(inhalation challenge with TDI–GSA conjugate)*					
0 (control)	>50 (8)	0/8	8	0	0
0.1	3200 (2)	6/6	5	2	1
	12 800 (4)				
*Laboratory 2***					
0 (control)	<50 (8)	0/8	8	0	0
0.1	3200 (3)	12/12	12	0	0
	12 800 (6)				
	51 200 (3)				

ELISA: IgG1 antibody titre, dilution^{-1} (number of animals).

corn oil. On day 22 one group was given an inhalation challenge with 0.13–2 p.p.m. TDI vapour and the second group was exposed to an aerosol containing 22 mg/m^3 TDI–GSA conjugate. A summary of the results is given in Table 6. The serum samples from all the animals in each of the treatment groups contained IgG1 TDI-specific antibodies as measured by PCA and by ELISA analysis, the ELISA antibody titres ranging from 3200 to 51 200 for the first treatment group and from 3200 to 12 800 for the second treatment group. Again, as in the experiment carried out by Laboratory 2, there was no pulmonary reaction in any of the treated animals on inhalation challenge with TDI vapour. However, a pulmonary reaction was observed in 3 out of 8 of the treated animals challenged with TDI–GSA conjugate and one of these reactions was in the severe response category.

3.5. Dinitrochlorobenzene (DNCB)

*Laboratory 1**. Four groups of guinea pigs were injected intradermally on day 1 with concentrations of DNCB in corn oil ranging from 0.3% to 3%. Each group of animals was exposed to the same inhalation challenge concentration of DNCB (achieved concentration 6.5–14.5 mg/m^3) on day 22. A summary of the results is given in Table 7. No IgG1 DNCB-specific antibodies were detected in any of the serum samples from the animals in the groups sensitised with 0.3% and 1% DNCB as measured by PCA analysis. However, IgG1 DNCB-specific antibodies were detected in 2 out of 7 of the serum samples from the animals in the group sensitised with the higher concentration of 3% DNCB. There was little difference in the IgG1 antibody titres for each of the treatment groups as measured by ELISA analysis with the maximum antibody titre in any of the serum samples being only 2560. A pulmonary reaction was observed in only one of the guinea pigs in each of the groups sensitised with 0.3% and 3% and these reactions were both in the severe response category. However, these responses were more consistent with irritant effects, with a slow decrease in respiratory rate, rather than specific pulmonary

Table 7
Summary of the serological and pulmonary results following sensitisation with DNCB

Sensitisation dose (%)	Antibody analysis		Pulmonary response category (number of animals in each category)		
	ELISA titre (serum dilution^{-1})	PCA result (no. of animals positive)	No effect	Moderate response	Severe response
*Laboratory 1**					
0 (control)	<10 (8)	0/8	8	0	0
0.3	<10 (2) 10 (3) 40 (2) 160 (1)	0/8	7	0	1
1.0	160 (2) 640 (5) 2560 (1)	0/8	8	0	0
3.0	160 (1) 640 (3) 2560 (3)	2/7	6	0	1
*Laboratory 2***					
0 (control)	<50 (8)	0/8	6	0	2
0.05	<50 (10) 50 (2)	0/12	9	0	3

ELISA: IgG1 antibody titre, dilution^{-1} (number of animals).

Table 8
Summary of the mass median aerodynamic diameters (MMADs) achieved in the exposure chamber during inhalation challenge

Test material	MMAD range achieved by Laboratory 1* (μm)	MMAD range achieved by Laboratory 2** (μm ± S.D.)
TMA	2.71–6.98	3.81 ± 1.73
MDI	1.01–1.64	2.17 ± 2.19
PHA (experiment 1)	3.79–4.81	5.92 ± 1.64
PHA (experiment 2)	0.61–18.02	4.71 ± 1.62
DNCB	2.19–12.70	2.17 ± 2.28

reactions where a sharp decrease in respiratory rate is more typical (e.g. as for the responses observed with TMA, MDI and PHA).

*Laboratory 2***. A group of guinea pigs was injected intradermally on day 1 with 0.1% TDI in 6% acetone in corn oil. The animals were exposed to a nominal challenge concentration of 2 p.p.m. estimated from the bubbler settings used in preliminary studies to achieve this concentration (the actual concentration was not measured because of problems caused by coating of the lens in the analyser) on day 22. A summary of the results is DNCB-specific antibodies were detected in 2 out of 12 of the animals in the treatment groups by ELISA analysis but the antibody titre for these animals was very low. A pulmonary reaction was observed in 3 out of 12 of the animals in the treatment group and these reactions were all in the severe response category. However, severe pulmonary responses were also observed in 2 out of 8 of the control animals, presumably due to an irritant response to DNCB.

The mass median aerodynamic diameters achieved in the exposure chambers at each inhalation challenge are summarised in Table 8.

4. Discussion

This investigation was designed to explore whether the intradermal injection of low molecular weight chemicals into guinea pigs, followed by the analysis of circulating homocytotropic antibodies and pulmonary responses to an inhalation challenge with the free chemical, might provide a means of assessing the potential of these materials to induce respiratory allergy in man. In the previous investigation (Botham et al., 1989), the intradermal injection of TMA was found to be capable of inducing high titre TMA-specific homocytotropic antibodies in the guinea pig. The subsequent inhalation challenge of the sensitised animals with both free TMA and TMA–protein conjugate was then able to elicit pulmonary responses in these animals. In contrast, DNCB was able to induce contact sensitisation but produced only low titre antibodies and was unable to elicit a pulmonary response at inhalation challenge with either free chemical or DNCB–protein conjugate.

The present investigation involved two collaborating industrial research laboratories, Zeneca Central Toxicology Laboratory and Unilever Environmental Safety Laboratory. The investigation was carried out using four recognised respiratory allergens (TMA, MDI, PHA and TDI) and DNCB, which is a potent contact allergen but does not cause respiratory allergy (Botham et al., 1989).

The results of this investigation demonstrated that the intradermal injection of the four known respiratory sensitisers was able to induce high titre antigen-specific homocytotropic antibodies in guinea pigs. In contrast, intradermal injection of the control material, DNCB, produced only very low titre antibodies and in a smaller proportion of the treated animals. Inhalation exposure to three of the four respiratory allergens — TMSA, MDI and PHA — approximately 3 weeks after sensitisation with the appropriate material induced a pulmonary response. However, no pulmonary responses were observed when animals which had been sensitised to TDI were exposed to TDI vapour at inhalation challenge. This may be attributable to the low level of TDI used at the in-

halation challenge (0.13–2 p.p.m.) which may have been inadequate for the induction of a pulmonary response. However, the use of higher inhalation challenge concentrations of TDI resulted in a respiratory response in both treated and control animals consistent with an irritant reaction. Alternatively, the reactivity of TDI may prevent it reaching and reacting with the appropriate target sites in the guinea pig respiratory tract. A pulmonary response was eventually demonstrated in a number of TDI-sensitised animals but only when a TDI–GSA conjugate preparation was used as the inhalation antigen, a procedure advocated by other investigations (Karol et al., 1980; Botham et al., 1988; Sarlo and Clark, 1992). It would therefore appear that in order to elicit a pulmonary response, challenge with free chemical may only be appropriate for particulate materials and that for volatile chemicals, such as TDI, a protein conjugate may be necessary to induce a response. Further work using a wider range of chemicals would be required to confirm this.

In the previous investigation (Botham et al., 1989), the intradermal injection of TMA and DNCB was at a single concentration. In the present studies a range of sensitisation doses for TMA, MDI, PHA and also DNCB were used. The results demonstrated a sensitisation dose related increase in IgG1 antibody titres with TMA, MDI and PHA. In addition, a moderate increase in antibody titres was also observed with DNCB. It would appear that an upper threshold dose may exist for each material above which there is no further increase in the antibody titres. This is seen with MDI, where above the sensitisation dose of 1% there was no further increase in the antibody titres. Conversely, there is probably also a lower sensitisation dose threshold for each of the materials, below which no antibody production (and therefore, sensitisation) occurs. The sensitisation dose which represents the lower threshold for each material appears to be very low. For example, a sensitising dose of 0.0003% MDI was still able to induce antibody production in some animals.

In respect of the pulmonary responses, there is no obvious relationship between the sensitisation dose and the pulmonary reactions. However, it would appear that there is probably a threshold sensitisation dose for each of the materials associated with the induction of a pulmonary response. As seen with MDI, where pulmonary responses were not observed below a sensitisation dose of 0.01%, this threshold is much higher than the threshold required for the induction of antibody production. An antibody response provides the potential for the elicitation of a pulmonary response to occur; however, there is no clear relationship between antibody titre and pulmonary responsiveness or indeed the severity of any pulmonary response. This is illustrated in the group of animals sensitised with 0.003% TMA, where two of the animals showed pulmonary reactions in the severe response category although the antibody titres for the animals in this group were relatively low, ranging from 10 to 640. In contrast, animals sensitised to a higher concentration of TMA produced antibody titres of up to 51 200 but did not respond on inhalation challenge. In addition, there was no clear relationship between the inhalation challenge concentration and pulmonary responsiveness, as seen with TMA.

In conclusion, this interlaboratory validation has confirmed the results of the previous investigation (Botham et al., 1989). The results were consistent between the two collaborating laboratories. However, although the assay shows promise as a means of identifying chemical respiratory sensitisers the use of this model in routine screening for the prediction of the ability of materials to induce respiratory allergy in man is probably questionable. The four known respiratory allergens were all able to induce high titre specific antibodies as measured by ELISA analysis compared with the control material, DNCB, which produced very low titre antibodies in only a small proportion of the treated animals. However, the inhalation challenge phase of this protocol was less conclusive, with only three of the four test materials — TMA, MDI and PHA — being able to induce a pulmonary response on exposure to the free chemical. Pulmonary responses were produced in the TDI sensitised animals but this required inhalation exposure to a TDI–protein conjugate. The problems encountered with the inhalation challenge due to the reactivity of some small molecular weight materials (for example, PHA which

must be freshly ground in order to induce a pulmonary response and TDI which requires conjugation to a protein in order to induce a pulmonary response), may result in the need for extensive preliminary experimentation to optimise the inhalation challenge conditions and this may not be practical for a routine toxicological test. Therefore, the induction of high titre antibodies to a material in guinea pigs following a single intradermal injection is probably a more useful aid in the prediction of the ability of that material to induce respiratory sensitisation. However, similar investigations (Dearman et al., 1992) are currently evaluating the use of a mouse model and the ability of low molecular weight materials to elevate total serum IgE levels as a predictive test, and the merits of each of these methods would require further investigation and comparison using a larger number of materials.

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