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Sensitisation of guinea pigs by inhalation exposure to low molecular weight chemicals

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SUMMARY

Guinea pigs could be immunologically sensitised (as shown by the development of antigen-specific homocytotropic antibodies) to toluene diisocyanate by exposing them for 3 h a day for 5 consecutive days to atmospheres containing free chemical. Pulmonary reactions could be elicited in many of the sensitised animals by challenging them with atmospheres containing protein conjugates of the chemical and then measuring changes in respiratory rate. Successful elicitation of pulmonary reactions appeared to depend upon a number of factors, including the quality of the protein conjugate used for the challenge, but possibly also the development of IgE as well as IgG1 antibodies. Antigen-specific homocytotropic antibodies were detected in guinea pigs similarly exposed by inhalation to two non-isocyanate respiratory allergens, trimellitic anhydride and a reactive dye. Although the animals were immunologically sensitised to the chemicals, challenge with atmospheres containing appropriate chemical-protein conjugates failed to stimulate changes in respiratory rate.

INTRODUCTION

The development of rhinitis and asthma following occupational exposure to dusts, vapours and gases has been recognised for many years [1]. The problem was

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Abbreviations: TDI, toluene diisocyanate; TMA, trimellitic anhydride; YMX4R, Procion Yellow MX4R; GPSA, guinea pig serum albumin; HDI, hexamethylene diisocyanate; UV, ultraviolet; HPLC, high performance liquid chromatography; PCA, passive cutaneous anaphylaxis assay; ELISA, enzyme-linked immunosorbent assay; MMAD, mass mean aerodynamic diameter.

first associated with exposure to small molecules (<1500 Da) in the 1940s following the introduction of plastics and the subsequent large-scale use of reactive chemicals such as diisocyanates and acid anhydrides. It is now known that occupational respiratory disease can be induced by a number of other small molecules, including several drugs (most notably antibiotics) and certain metal salts and dyestuffs (see [2,3] for a comprehensive listing of causative agents).

In many cases of occupational rhinitis and asthma, re-exposure to the causative agent, even at relatively low concentrations, results in the development of symptoms, usually within minutes of exposure, although occasionally not until several hours later. This pattern of symptoms is highly suggestive of an immunological pathogenesis and in particular of an IgE-mediated (type I hypersensitivity) response. Evidence is increasing that in certain individuals symptoms associated with exposure to low molecular weight chemicals can be mediated by such a mechanism. Specific IgE antibodies have been demonstrated by radioimmunoassay of serum or by skin testing individuals whose symptoms were associated with exposure to isocyanates [4-7], acid anhydrides [8-10], platinum salts [11,12] and reactive dyes [13,14]. However, in many cases of isocyanate-induced rhinitis and asthma it has not been possible to detect specific antibodies and this has led to speculation that isocyanates can also induce respiratory symptoms by non-immunological mechanisms [15].

A number of potential animal models of respiratory allergy to low molecular weight materials have been investigated. Many have involved sensitising animals either by inhalation exposure to a protein conjugate of the chemical [16,17] or by the injection or topical application of free or conjugated chemical, with or without adjuvants [18-20]. However, Patterson et al. [21] were able to induce immediate-type airway responses in dogs which had been sensitised by instillation into the lung of an aerosol of toluene diisocyanate (TDI). Pulmonary responses correlated with skin test reactivity to the chemical.

Sensitisation of guinea pigs to inhaled TDI was achieved by Karol et al. [22] using an inhalation exposure protocol. Animals were exposed to the chemical for 3 h a day for 5 consecutive days; following an exposure-free period of 2 weeks, sensitisation could be elicited by challenging with an atmosphere containing a protein conjugate of TDI and measuring the subsequent increase in respiratory rate. An immunological mechanism for this effect was confirmed by the detection of IgE and IgG1 anti-TDI antibodies in the sera of the animals.

We have evaluated the utility of this protocol using not only TDI, but also two other human sensitisers, trimellitic anhydride (TMA) and Procion Yellow MX4R (YMX4R), a dichlorotriazine-based reactive dye. The overall aim of this work was to determine whether a protocol of this type could form the basis of a predictive toxicological test for low molecular weight respiratory allergens.

MATERIALS AND METHODS

Animals

Female Dunkin-Hartley, albino guinea pigs with a weight range of 237–441 g were used throughout the studies. The animals were obtained from Porcellus Animal Breeding, Heathfield, Sussex, U.K. and were housed one or two per cage and allowed food and water ad libitum except during exposure periods.

Chemicals

Toluene diisocyanate was obtained from ICI Organics Division, Manchester, U.K., as an 80:20 mixture of the 2,4- and 2,6-isomers (known as SUPRASEC EN) and *p*-tolyl isocyanate was from Kodak Chemicals, Liverpool, U.K. Hexamethylene diisocyanate and trimellitic anhydride were obtained from Aldrich Chemical Co., Gillingham, U.K. The dichlorotriazine reactive dye (YMX4R) (Fig.1) was supplied by ICI Organics Division. Guinea pig serum albumin was obtained from Sigma Chemical Co., Poole, U.K. Nitrogen dioxide was supplied by Cambrian Gases as a 0.1% (v/v) dilution in air.

Preparation of hapten-protein conjugates

TDI. Conjugates of TDI with guinea pig serum albumin (GPSA) were produced by three different methods in order to vary the degree of substitution (hapten density) and also the extent of cross-linking of the protein by TDI: 100 mg of TDI were incubated with 500 mg of GPSA in 8 ml of H₂O, (i) for 30 min at 27°C, (ii) for 3 h at 4°C, (iii) for 3 h at 27°C. In addition, a conjugate was prepared by incubating 100 mg of *p*-tolyl isocyanate with 500 mg of GPSA in 8 ml of H₂O for 1 h at 27°C. Hexamethylene diisocyanate (HDI) was conjugated to GPSA by incubating 100 mg of chemical with 500 mg of protein in 8 ml of H₂O for 30 min at 4°C. Each conjugate was desalted on a Sephadex G-25 column and freeze dried. The degrees of substitution of the TDI-GPSA conjugates were assessed by measuring the absorption of a solution of conjugate in water at 242 nm and were, (i) 15, (ii) 30, (iii) 49 mol·mol⁻¹ respectively. A further conjugate of TDI-GPSA was obtained from Dr. M. Karol, University of Pittsburg, PA, U.S.A. and this had a degree of substitution of 50 mol·mol⁻¹.

TMA. TMA was dissolved in dioxan (125 mg·ml⁻¹) and 0.35 ml was added in 0.05 ml aliquots to 150 mg of GPSA in 5 ml of H₂O over a 1 h period. The solu-

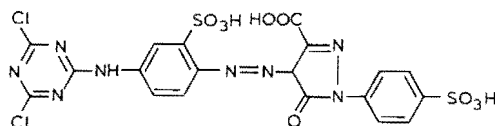


Fig. 1. Structure of the dichlorotriazine reactive dye Procion Yellow MX4R.

tion was stirred overnight at 27°C and at a pH of 7.6. The conjugate was desalted on a Sephadex G-25 column, dialysed against 0.1 M ammonium acetate buffer, pH 7.6, and then freeze dried. The degree of substitution was assessed by UV spectrometry as described by Zeiss et al. [23] and was found to be 21 mol·mol⁻¹.

YMX4R. YMX4R was dissolved in H₂O (250 mg·ml⁻¹) and 0.5 ml was added to 360 mg of GPSA in 12 ml H₂O over a 1 h period. Incubation was continued for a further 3 h at 27°C and at pH 8.0. The solution was then desalted on a Sephadex G-25 column and the high molecular weight material was dialysed against H₂O. The λ_{\max} of ethanolamine-deactivated dye was found to be 430 nm and the absorption of the conjugate at this wavelength was used to determine the degree of substitution (which was 5 mol·mol⁻¹).

Atmosphere generation and analysis

TDI. Atmospheres of TDI vapour were generated for the sensitisation exposures using a sintered glass bubbler maintained at 35°C. The atmospheres were analysed by a method based on that of Dunlap et al. [24]; atmospheres were drawn through sintered glass impingers containing a solution of *N*-4-nitrobenzyl-*N*-*n*-propylamine in toluene in order to trap any isocyanates present as urea derivatives. These were subsequently quantified by HPLC. The challenge exposures of TDI-protein conjugates were generated from 1% aqueous solutions of TDI-GPSA using a Wrights nebuliser and atmospheric concentrations were determined gravimetrically using VM-1 25 mm open-faced filters (Gelman S, Northampton, U.K.).

TMA and YMX4R. Atmospheres were generated for the sensitisation exposures using a Wrights Dust Feed mechanism (Adams, London, U.K.). The challenge exposures of protein conjugates were generated as for TDI. The particulate concentrations of the sensitisation and challenge atmospheres were measured gravimetrically, again as for TDI. The particle size distributions were determined using a cascade impactor (Marple cascade impactor, Schaeffer Instruments, Wantage, U.K.).

NO₂. NO₂ was diluted with clean air to provide a final concentration of 50 ppm.

All atmospheres were sampled in the breathing zone of the exposed animals.

Guinea pigs were exposed nose-only to the atmospheres which were generated into circular Perspex exposure chambers having an internal volume of approximately 40 litres.

Measurement of pulmonary hypersensitivity

It has been demonstrated by a number of investigators, including Karol et al. [22], that guinea pigs which have been sensitised immunologically to an antigen may res-

pond to an inhalation challenge with the antigen with an increase in respiratory rate. Respiratory rate was monitored continuously during the challenge procedures by standard plethysmographic techniques. The typical challenge regimen was a settling period, usually 5–10 min, a minimum of 5 min for establishing the control rate of respiration, followed by a 15 min challenge with an atmosphere containing hapten-GPSA conjugate. After challenge the animals were allowed to recover and their respiration rate was monitored for a further 15–20 min. During the settling, control and recovery periods animals were exposed to an aerosol of distilled water to accustom them to the noise produced by the Wrights nebuliser.

Detection of antibodies in guinea pig serum

(a) *Passive cutaneous anaphylaxis (PCA) assay.* Serum from exposed and control animals was diluted serially in saline and then injected intradermally into the shaved flanks of naive guinea pigs. An appropriate hapten-GPSA conjugate (2.5 mg) together with Evans blue dye (5 mg) was injected intravenously in 0.5 ml saline either 6 h later (to detect IgG1 anti-hapten antibodies) or 6 days later (to detect IgE anti-hapten antibodies). The animals were killed and the cutaneous reactions were read on the inner surface of the skin 30 min after the i.v. injections, the antibody titre being the highest dilution of serum giving a measurable blue lesion in the skin.

(b) *Enzyme-linked immunosorbent assay (ELISA).* This alternative assay for IgG1 anti-hapten antibodies involved coating plastic microtitre plates (Nunc Immunoplate type II, Nunc, Copenhagen, Denmark) with $1-10 \mu\text{g} \cdot \text{ml}^{-1}$ of hapten-GPSA conjugate by overnight incubation of the conjugate at 4°C in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Guinea pig serum was added to the coated plates at dilutions ranging from 1 in 15 to 1 in 50 and incubated for 2 h at 27°C . The plates were then incubated with rabbit anti-guinea pig IgG1 (Miles Scientific, Slough, U.K.) at a dilution of 1 in 2500 followed by peroxidase-labelled goat anti-rabbit IgG (Miles Scientific) at a dilution of 1 in 5000, both incubations being for 2 h at 27°C . Substrate (*o*-phenylenediamine) was added for 10 min and then the reaction was stopped with 0.5 M citric acid. Absorbance was measured at 450 nm by means of a Multiskan (Flow Laboratories, Irvine, Scotland, U.K.). Between each incubation the plates were washed with phosphate-buffered saline containing 0.5% Tween 20 (PBS-Tween), a buffer which was also used to prepare the dilutions of the sera and antisera.

RESULTS

Sensitisation with toluene diisocyanate

Four groups of guinea pigs, each containing ten animals, were exposed to target atmospheric concentrations of 0, 1, 3 and 4 ppm TDI for 3 h a day for 5 consecutive

TABLE I

ANTI-TDI ANTIBODY TITRES IN THE SERA OF GUINEA PIGS EXPOSED TO TDI BY INHALATION

Sera were tested in PCA assays.

Sensitising dose of TDI (ppm)	Anti-TDI antibody titres (serum dilution ⁻¹)	
	IgG1	IgE
0	No antibodies detected	
1	1024 (<i>n</i> = 7)	16 (<i>n</i> = 3)
	256 (<i>n</i> = 1)	4 (<i>n</i> = 1)
	64 (<i>n</i> = 2)	0 (<i>n</i> = 2)
		NT (<i>n</i> = 4)
3	1024 (<i>n</i> = 8)	16 (<i>n</i> = 1)
	256 (<i>n</i> = 2)	4 (<i>n</i> = 5)
		0 (<i>n</i> = 2)
		NT (<i>n</i> = 2)
4	1024 (<i>n</i> = 8)	4 (<i>n</i> = 2)
	256 (<i>n</i> = 2)	0 (<i>n</i> = 8)

NT, not tested.

days (the analysed dose levels were 0, 1.29, 3.10 and 4.24 ppm). These concentrations were selected on the basis of the concentrations employed by Karol [25] and the known toxicity of TDI. Two weeks later (day 19) serum from each animal was analysed for IgG1 and IgE anti-TDI antibodies using a PCA assay. In this assay the injected antigen was a TDI-GPSA conjugate which had been supplied by Dr. M. Karol, University of Pittsburg, PA, U.S.A. ('Karol' conjugate). Table I shows that high titres of IgG1 anti-TDI antibodies could be detected in the sera of all of the animals which had been exposed to TDI, confirming the plateau response seen by Karol [25]. In addition, IgE anti-TDI antibodies were present in the sera of a significant proportion of the animals, particularly those which had been exposed to 1 and 3 ppm TDI.

Sera from three of the animals which had been sensitised by exposure to 1 ppm TDI were then used to study the specificity of the IgG1 antibody response and the influence on antibody titre of the quality of the TDI-GPSA conjugate used in the PCA assay (Table II). Low titres of IgG1 antibody were detected using a protein conjugate of hexamethylene diisocyanate (HDI-GPSA), suggesting that a small proportion of the antibody response may have been directed against new antigenic determinants on the hapten-modified GPSA, but that the major response was against the sensitising hapten, TDI. Using five different TDI-GPSA conjugates, IgG1 anti-TDI antibody titres were found to vary considerably; the highest titres were detected using two TDI-GPSA conjugates which had been prepared by in-

TABLE II

THE SPECIFICITY OF THE ANTIBODY RESPONSE TO INHALED TDI AND THE INFLUENCE ON ANTIBODY TITRE OF THE QUALITY OF THE PROTEIN CONJUGATE USED IN THE ASSAY

Conjugate used in PCA assay	Degree of substitution (mol · mol ⁻¹)	IgG1 antibody titres (serum dilution ⁻¹)		
		Animal No.114	Animal No.116	Animal No.119
'Karol' TDI-GPSA	50	1024	1024	1024
'ICI' (i) TDI-GPSA	15	128	128	128
'ICI' (ii) TDI-GPSA	30	256	2048	2048
'ICI' (iii) TDI-GPSA	49	128	128	128
'ICI' <i>p</i> -tolyl-GPSA	~30	256	2048	NT
HDI-GPSA	ND	8	32	8

ND, not determined; NT, not tested.

cubating TDI with protein at 4°C in order to minimise the extent of cross-linking of protein by the bifunctional isocyanate (these were the 'Karol' conjugate and 'ICI' conjugate (ii)). Similar high titres were detected using a conjugate which had been prepared using the monofunctional *p*-tolyl isocyanate. The detection of antibodies may therefore depend not only on the degree of substitution of the conjugate used in the assay, but also, and perhaps more importantly, on minimising changes in protein structure due to cross-linking, an effect which may reduce the accessibility of hapten to antibody.

On days 22, 23, 24, 29 and 30 the animals were challenged by exposure to an atmosphere containing either the 'Karol' TDI-GPSA conjugate or the 'ICI' conjugate (ii). The atmospheric concentration of the conjugate ranged from 18 to 90 mg · m⁻³, but this variation did not influence the responses of the guinea pigs. The results are shown in Fig. 2 as the maximum increase in respiratory rate following any one of the five challenges, expressed as a percentage of the mean increase in respiratory rate of challenged control animals (i.e. those animals which had not previously been exposed to TDI). The line above which Karol et al. [22] considered a respiratory rate change to be significant, namely three standard deviations above the mean control value, is shown in this figure.

Seven out of nine animals exposed to 1 ppm TDI showed a significant increase in respiratory rate when challenged with the 'Karol' conjugate on days 22–24. There were no significant increases in rate when the same animals were challenged with the 'ICI' conjugate on days 29 and 30.

Nine out of ten animals sensitised with 3 ppm TDI responded to respiratory challenge with 'Karol' conjugate on days 22–24, but again the animals did not respond to the 'ICI' conjugate on days 29 and 30.

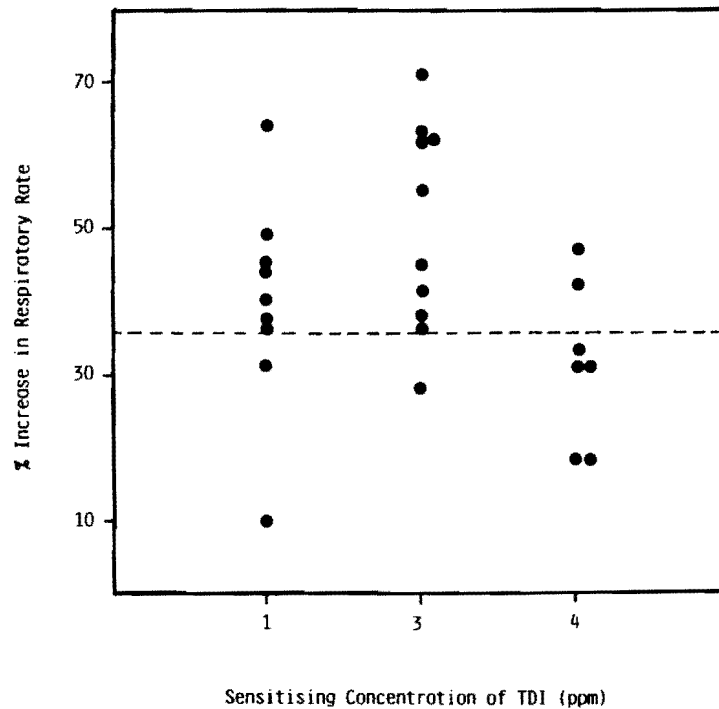


Fig. 2. Increase in respiratory rate following challenge of TDI-sensitised animals with an atmosphere containing TDI-GPSA. The data are expressed as the percentage increase in rate for each individual animal compared with the mean increase in rate of the challenged control group; the maximum increase in rate following any one of two or three challenges with 'Karol' conjugate is shown. Animals sensitised with 0 (controls) or 4 ppm TDI were challenged on days 29 and 30; animals sensitised with 1 or 3 ppm TDI were challenged on days 22-24. The dashed line represents three standard deviations above the mean increase in rate of the challenged control group; increases in rate above this line are considered to be significant [22].

The 4 ppm dose group showed no significant effects when challenged with the 'ICI' conjugate on days 22-24. However, when these animals were challenged on days 29 and 30 with the 'Karol' conjugate, two out of seven animals responded.

Control animals were challenged with the 'ICI' conjugate on days 22-24 and the 'Karol' conjugate on days 29 and 30 and no reactions were noted.

This experiment indicated that the physico-chemical properties of the hapten-protein conjugate are crucial, not only to the detection of antibodies but also to the elicitation of pulmonary hypersensitivity responses. This was confirmed when the experiment was repeated; although high titres of IgG1 anti-TDI antibodies were found by PCA in the sera of animals exposed to TDI, IgE antibodies could not be detected and no pulmonary reactions were seen following challenge with TDI-GPSA (data not shown). In this experiment 'ICI' conjugate (ii) was used throughout.

Sensitisation with trimellitic anhydride

Four groups of guinea pigs (8-12 animals per group) were exposed to at-

mospheres containing trimellitic anhydride for 3 h a day for 5 consecutive days. The target concentrations of TMA were 0, 1, 15 and 100 $\text{mg} \cdot \text{m}^{-3}$ (measured concentrations were 0, 2.1, 14.2 and 108.9 $\text{mg} \cdot \text{m}^{-3}$ with mass mean aerodynamic diameters (MMAD) ranging between 3.6 and 3.8 μm for target concentrations of 1 and 15 mg/m^3 ; no particle size analysis was performed on the atmosphere of target concentration 100 $\text{mg} \cdot \text{m}^{-3}$). These concentrations were based on the desire to use a range of concentrations, the highest of which induces mild lung toxicity (data not shown). Two weeks later (day 19) serum from each animal was analysed for IgG1 anti-TMA antibodies by both ELISA and PCA and for IgE anti-TMA antibodies by PCA only. The majority of the animals were then challenged on days 22 and 29 by exposure to an atmosphere containing TMA-GPSA (concentration range 5–18 $\text{mg} \cdot \text{m}^{-3}$). Four animals from the groups exposed to 15 and 100 $\text{mg} \cdot \text{m}^{-3}$ TMA were not challenged. On day 30 serum from all of the animals was again analysed for anti-hapten antibodies. All antibody assays and pulmonary challenges were performed with a single TMA-GPSA conjugate which had a degree of substitution of 21 $\text{mol} \cdot \text{mol}^{-1}$.

The results of the antibody assays are shown in Fig. 3. Before challenge, IgG1 anti-TMA antibodies could be detected using the ELISA procedure in the majority

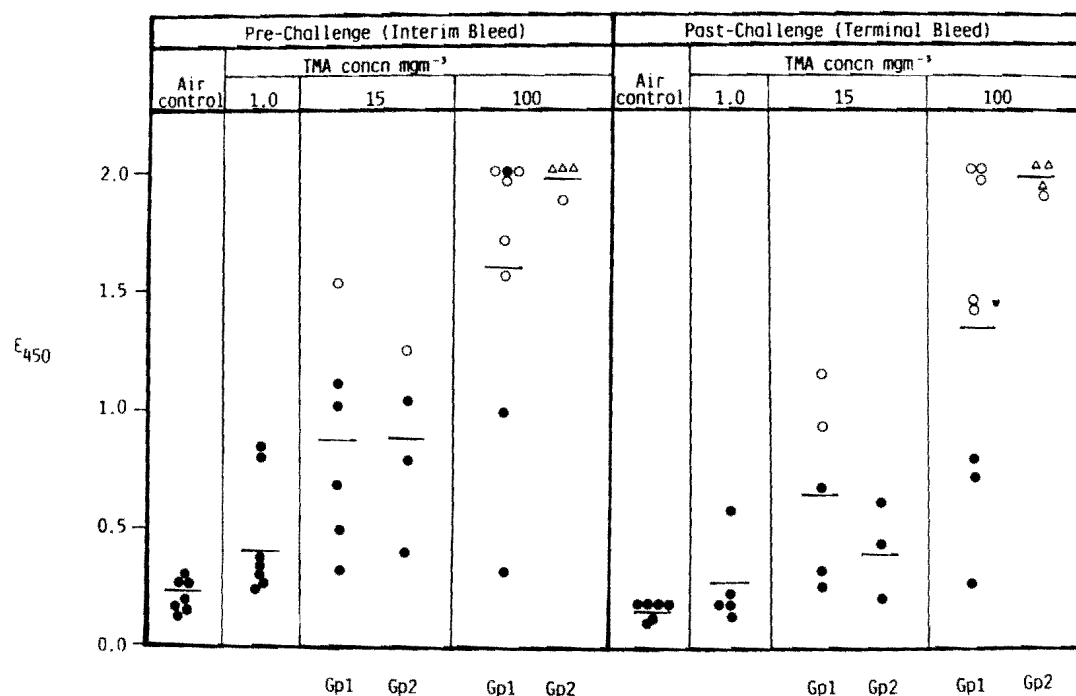


Fig. 3. IgG1 and IgE anti-TMA antibodies in the sera of guinea pigs sensitised by inhalation exposure to TMA. (●) The ELISA titre of IgG1 antibody for each individual serum, expressed as an E_{450} ; a serum dilution of 1 in 50 was used. (○) These sera were confirmed as IgG1-positive using a PCA assay in which a serum dilution of 1 in 2 was used. (Δ) These sera were both IgG1- and IgE-positive in PCA assays. For the animals exposed to 15 and 100 $\text{mg} \cdot \text{m}^{-3}$ TMA, group 1 was challenged with TMA-GPSA but group 2 remained unchallenged.

of animals exposed to 15 or 100 mg·m⁻³ TMA but only in two animals exposed to 1 mg·m⁻³ TMA. PCA assays confirmed that nine of the 12 animals exposed to 100 mg·m⁻³ TMA were IgG1-positive and that three of these sera were also IgE-positive. However, only two of the ten animals exposed to 15 mg·m⁻³ were antibody-positive in the PCA assay. This may indicate that the ELISA procedure is more sensitive than the PCA assay or that the anti-guinea pig IgG1 antibody used in the enzyme immunoassay has some cross-reactivity with non-homocytotropic subclasses of IgG antibody.

Challenge with an atmosphere of TMA-GPSA resulted in no significant increases in respiratory rate in any of the animals. Following this challenge, antibody titres were slightly lower, but the challenge procedures themselves had little effect on antibody titre (in Fig. 3, compare groups 1 and 2 for the animals sensitised by exposure to 15 or 100 mg·m⁻³ TMA; animals in groups 2 did not receive a challenge with TMA-GPSA).

The hapten specificity of the antibody response was confirmed using ELISA assays in which plates were coated with an irrelevant conjugate, TDI-GPSA; in these control assays the absorbances of all the sera from TMA-exposed animals were equivalent to those of the control sera from unexposed animals.

Sensitisation with dichlorotriazine-reactive dye (YMX4R)

Three groups of guinea pigs (eight animals per group) were exposed to target atmospheric concentrations of 0, 10 or 100 mg·m⁻³ reactive dye (measured concentrations were 0, 14.7 and 94.0 mg·m⁻³ with MMAD ranging between 4.4 and 4.8 μm) for 3 h per day for 5 consecutive days. Half of the animals in each group were exposed for 1 h to 50 ppm NO₂, 2 h before the first exposure, in order to determine whether exposure to a mild pulmonary irritant may enhance the development of sensitisation by, for example, increasing the permeability of the lung tissue to the hapten.

On day 21, serum was analysed for anti-YMX4R antibodies and this was repeated on day 30 following challenges on days 22, 23 and 29 with YMX4R-GPSA. For these experiments a single conjugate was used and this had a degree of substitution of 5 mol·mol⁻¹.

ELISA assays showed that sera from about two-thirds of the animals exposed to 10 mg·m⁻³ reactive dye were positive before challenge for IgG1 anti-YMX4R antibody (Fig. 4). None of these sera was positive in PCA assays. At the higher concentration of 100 mg·m⁻³, the majority of the sera were positive in both assays, with three of the sera being positive also for IgE antibody. Pre-exposure of animals to NO₂ appeared to have little effect on antibody titre or on the development of an IgE response.

When the animals were challenged with conjugated hapten (concentration range 3–52 mg·m⁻³), no significant changes in respiratory rate were detected in any of the animals at any of the three challenges. Antibody titres were little changed

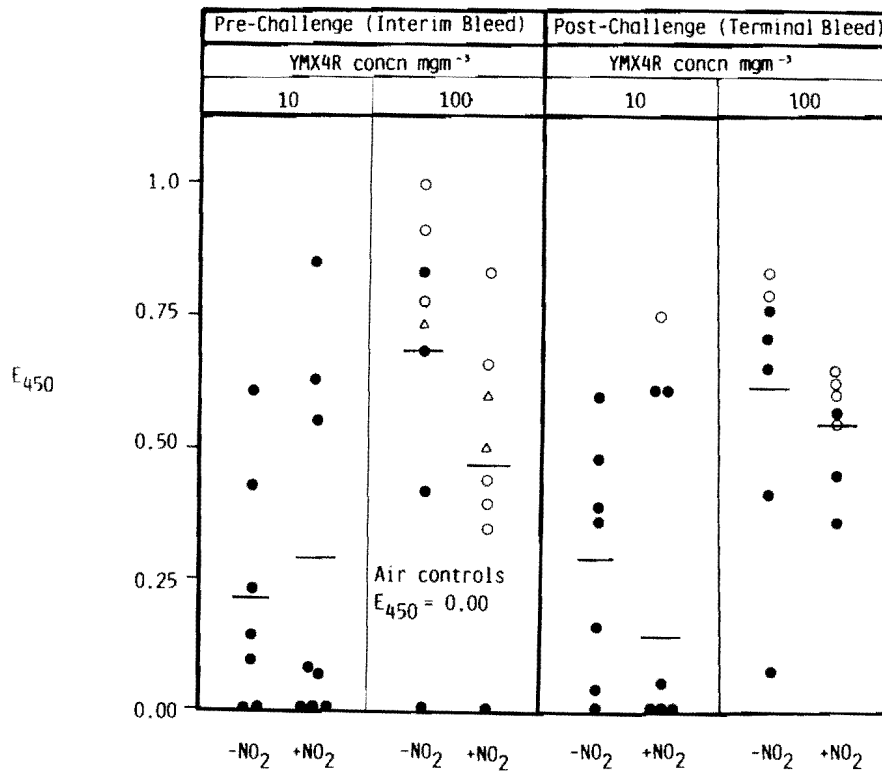


Fig. 4. IgG1 and IgE anti-dye antibodies in the sera of guinea pigs sensitised by inhalation exposure to YMX4R, a dichlorotriazine reactive dye. Certain animals were exposed to 50 ppm NO₂ 2 h before the initial exposure to the dye, as indicated. The symbols represent ELISA and PCA assay titres as in Fig. 3; ELISA assays were performed at a serum dilution of 1 in 15.

following the challenges, except that at this time no IgE-positive sera could be detected.

DISCUSSION

There have been few reports of the induction of pulmonary hypersensitivity in experimental animals following inhalation exposure to unconjugated low molecular weight chemicals. The most significant progress has been made by Karol et al. [22], who succeeded in sensitising guinea pigs to TDI and *p*-tolyl isocyanate (TMI), as evidenced by the development of hapten-specific homocytotropic antibodies, by exposing them daily for 5 consecutive days to TDI or TMI vapour. In a later study [25] pulmonary hypersensitivity was elicited in TDI-sensitised animals by exposing them to a conjugate of TDI with homologous protein (GPSA), the response being monitored by measuring the increase in respiratory rate following challenge.

However, the administration of low molecular weight chemicals to the lung does not inevitably lead to the development of sensitisation. Thus, Parker and Turk [26] found that intratracheal intubation of guinea pigs with the metal salts potassium

dichromate and nickel sulphate (both of which have been shown to cause occupational asthma in humans [27]) resulted in inhibition of the induction of contact sensitisation to the salts. The inhibition to either agent was hapten-specific and it was suggested that it was due to the development of a specific immunological unresponsiveness or tolerance. Similarly, Doe et al. [28] found that exposure of guinea pigs to TDI by inhalation resulted in the development of a hapten-specific inhibition of the induction of contact sensitivity to TDI. Pre-treatment of animals with cyclophosphamide not only prevented the development of tolerance but also allowed the inhalation exposure alone to sensitise the animals, as shown by subsequent dermal challenge. It was proposed, but not formally demonstrated, that the tolerance was mediated by suppressor cells. A similar phenomenon has been studied by Holt and his co-workers (reviewed in [29]) who found that in mice and rats, repeated inhalation of aerosols containing high molecular weight materials, such as ovalbumin, resulted initially in the development of an IgE antibody response to the protein. However, repeated exposure initiated an expansion within lymph nodes draining the upper respiratory tract of antigen- and IgE isotype-specific suppressor cells which down-regulated the IgE response.

The studies reported in this communication were performed to further an overall aim of developing a predictive animal test for low molecular weight respiratory allergens. It was felt that such a model should not only mirror human exposure as closely as possible, but should also have the ability to assess the possible development of tolerance as described above. Consequently, in the experiments reported, animals were exposed to unconjugated chemicals exclusively by the inhalation route.

The results showed that sensitisation to TDI could be induced by exposure to the chemical as a vapour and that pulmonary reactions could be elicited in sensitised animals by challenging with TDI-GPSA, thus confirming the findings of Karol et al. [22]. Both the antibody and pulmonary responses seen with TDI were critically dependent upon the quality of the hapten-protein conjugate used in the PCA assay or as the challenge antigen. The isocyanate moieties on TDI are highly reactive under physiological conditions with nucleophilic groups, including those found on proteins (e.g., $-NH_2$ on lysine, $-SH$ on cysteine and $-OH$ on serine). Thus, a diisocyanate such as TDI will not only react with protein at many sites but will also cause cross-linking of the molecule. The resulting change in protein conformation may result in haptenic groups becoming sterically hindered from interaction with antibody, thus affecting both antibody titre and the potential for *in vivo* pulmonary responses to the conjugate. A similar critical dependence on conjugate quality has been found in diagnostic studies of humans exposed to isocyanates. A number of investigators have found that the ability to detect human IgE anti-TDI antibodies in radioallergosorbent or skin tests is increased using conjugates prepared from monoisocyanates (reviewed in [30]).

In our studies, an attempt was made to minimise cross-linking by preparing a conjugate of TDI at a lowered temperature ($4^\circ C$) and also by using a conjugate of a

mono-functional isocyanate, *p*-tolyl isocyanate. These conjugates, together with a TDI conjugate prepared by Dr. M. Karol (also at 4°C) detected the highest titres of IgG1 anti-TDI antibodies in the PCA assay. However, only the 'Karol' conjugate was able to elicit pulmonary hypersensitivity responses and the precise characteristics of the ideal conjugate for use both *in vitro* and *in vivo* are still not known.

The induction of sensitisation to TMA and to YMX4R, a dichlorotriazine reactive dye, represents the first report of sensitisation to non-isocyanate low molecular weight allergens in an animal model based on inhalation exposure to free hapten. However, elicitation of this sensitisation by challenging with atmospheres containing appropriate protein conjugates could not be achieved. This again may indicate that the conjugates used to challenge the animals were inadequate for *in vivo* interaction with antibody. For example, the degree of substitution may have been too low. However, in each case it was not possible to prepare a conjugate with a significantly higher hapten density, presumably because TMA and YMX4R are not as reactive with protein as TDI. An alternative explanation is that the sensitisation regimen employed did not allow the development of antigen-specific antibodies of the most appropriate isotype for *in vivo* interaction of antigen with antibody bound to mast cells in the lung. Although both IgG1 and IgE antibodies are capable of mediating immediate-type hypersensitivity reactions in guinea pigs [31], it is possible that pulmonary hypersensitivity reactions to chemicals may be mediated preferentially by antibodies of the IgE class which are able to remain fixed to mast cells for many weeks, rather than IgG1 antibodies which become detached from mast cells within 12–24 h [32]. It is thus of interest that in the first of the two experiments performed with TDI, the detection of respiratory rate changes following challenge with conjugate coincided with the detection of IgE anti-TDI antibodies in the majority of the sera taken from exposed animals. Very few animals sensitised by exposure to TMA and YMX4R were found to be IgE-positive and, in the case of YMX4R-sensitised animals, the IgE response appeared to be transient (Fig. 4).

In conclusion, we have been able to show that guinea pigs can be sensitised by inhalation exposure to three low molecular weight chemicals which are known to cause respiratory allergy in humans. However, it has been difficult to elicit pulmonary reactions following challenge of sensitised animals with chemical-protein conjugates. The reasons for this discrepancy are now being investigated. The likelihood that this kind of animal model could form the basis of a predictive toxicological screen for respiratory allergens must await the outcome of these and further investigations on the interaction of inhaled low molecular weight chemicals with the immune system.

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