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Antibody responses of rats after immunization with organic acid anhydrides as a model of predictive testing

by Xing-Dong Zhang, MD,¹ Hans Welinder, PhD,¹ Bo AG Jönsson, PhD,¹ Staffan Skerfving, MD¹

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Objectives The sensitizing properties of organic acid anhydrides (OAA) were evaluated in a rat model.

Methods The development of specific immunoglobulin (Ig) E and Ig G in serum was investigated after immunization with 14 OAA and 3 OAA conjugates. Brown Norway rats were injected intradermally with 0.1 ml of 0.2 M OAA in liquid paraffin or 1.4 mg of rat serum albumin conjugate in saline. Serum samples were collected after 4 weeks. Antibodies were analyzed with enzyme-linked immunosorbent assay.

Results The serum titers of specific Ig E after immunization with the different free OAA varied from <50 to 6400. The rats immunized with 4-methylphthalic anhydride exhibited the highest titers. The specificity of Ig E was demonstrated by enzyme-linked immunosorbent assay inhibition tests. A good correlation was observed between the Ig E and Ig G titers. Immunization with OAA conjugates showed results parallel to the findings for the free compounds. Importantly, the Ig E titers for the OAA agreed well with findings from guinea pigs and with literature data from epidemiologic studies of exposed workers.

Conclusions The present animal model may be a valuable tool for predicting the sensitizing potential of OAA and possibly the sensitizing potential of low-molecular-weight compounds in general. Furthermore, the antibody specificity of the haptens and the variations in the magnitude of the antibody titers indicate a valuable approach for studies of quantitative structure-activity relationships.

Key terms animal model, immunoglobulin E, immunoglobulin G, occupational allergy, structure-activity relationship.

Knowledge about the chemical determinants of inhalant allergens that cause immune responses has both theoretical and practical significance. Thus interest in predictive testing of allergenicity (1, 2) and in quantitative structure-activity relationships (3–6) is growing.

There are several approaches for testing allergenicity in chemicals. Wass & Belin (7) suggested that the in-vitro reactivity of chemicals with proteins can be used to predict their sensitizing properties. Sarlo & Clark (8) introduced a stepwise procedure to detect chemical allergens, including both subcutaneous and inhalation sensitization of guinea pigs. The alternative use of a rat bioassay was described by Pauluhn (6). Topical sensitization of mice followed by the registration of alterations of the serum concentration of immunoglobulin (Ig) E and differential cytokine production is another interesting approach (5, 9, 10). The induction of specific antibodies may be a sensitive indicator of respiratory sensitization (1).

Knowledge of structure-activity relationships for respiratory sensitizers may be an important tool in predicting the immunogenicity of chemicals (3). Thus a need exists for animal experimental data on relevant chemicals to be combined with chemical, physical, and epidemiologic information.

Organic acid anhydrides (OAA) are reactive chemicals that induce specific Ig E antibodies in exposed workers (11–14). Ig E is involved in the pathogenesis behind rhinoconjunctivitis and asthma in association with exposure to OAA. Thus OAA are good model compounds for studies of the relationship between chemical characteristics and the induction of specific antibodies. Guinea pigs intradermally immunized with 13 OAA showed a wide variation of induced Ig G₁ titers between the different OAA (15). However, while Ig G₁ is the main anaphylactic antibody in guinea pigs, Ig E is important for the immediate allergic responses in rats, as well as in humans (16, 17).

¹ Department of Occupational and Environmental Medicine, University Hospital, Lund, Sweden.

Reprint requests to: Dr Hans Welinder, Department of Occupational and Environmental Medicine, University Hospital, S-221 85 Lund, Sweden. [e-mail: hans.welinder@ymed.lu.se]

The aim of the present study was to evaluate antibody responses in rats after intradermal immunization with OAA as a model of predictive testing for allergenicity.

Material and methods

Animals

Male, inbred Brown Norway (BN) rats (Møllegaard Breeding Center Ltd, Denmark) were used. The strain origin was Zentralinstitut für Versuchstierzucht, Hannover, Germany. They weighed 100 to 150 g when obtained. The rats were acclimatized to the animal facilities for 1 week before the experiments. The microbiological monitoring of the animals and the animal facilities complied with the recommendations of the European Laboratory Animal Science Associations.

Ethics

The animal experiments were approved by the Animal Research Ethics Committee of the Lund University.

Chemicals

Succinic anhydride (SA), 4-methylhexahydrophthalic anhydride (4-MHHPA; *cis*- isomer with regard to the carboxyl groups and a mixture of *cis*- and *trans*- isomers with regard to the methyl group), and 1,2,3,6-tetrahydrophthalic anhydride (1,2,3,6-THPA) were obtained from Janssen Chimica (Geel, Belgium); meso-dimethylsuccinic anhydride (DMSA) was prepared from meso-dimethylsuccinic acid (Aldrich, Gillingham, United Kingdom) in our laboratory (identity and purity checked with gas chromatography with mass-spectrometric detection (GC-MS) and nuclear magnetic resonance; meso-diethylsuccinic anhydride (DESA) was obtained from Synthelec AB (Lund, Sweden); maleic anhydride (MA) and *cis*-hexahydrophthalic anhydride (*cis*-HHPA) came from Merck (Darmstadt, Germany); methylmaleic anhydride (MMA) and 3,4,5,6-tetrahydrophthalic anhydride (3,4,5,6-THPA) were from Sigma Chemical Co (St Louis, MO, USA); 4-methylphthalic anhydride (4-MPA) came from Aldrich-Chemie (Steinheim, Germany); 3,4-methyltetrahydrophthalic anhydride (3,4-MTHPA) and 4,4-methyltetrahydrophthalic anhydride (4,4-MTHPA) were prepared in our laboratory with identity and purity checked with GC-MS; phthalic anhydride (PA) was obtained from Mallinckrodt Inc (Paris, KY, USA); and trimellitic anhydride (TMA) came from Fluka Chemie AG (Buchs, Switzerland). The chemical structures of the OAA are shown in figure 1. The purity of the various OAA was >97%. However, DESA was found to isomerize during storage to the corresponding raceme form, which reduced the purity. Rat serum albumin (RSA, fraction V) was purchased from the Sigma Chemical Company (St Louis, MO, USA), and

liquid paraffin was obtained from Apoteksbolaget (Umeå, Sweden).

Preparation of conjugates between anhydrides and rat serum albumin

The conjugates were prepared by reacting the anhydrides with RSA. All the anhydrides were dissolved in water-free dioxane and dripped into a cooled (+4 to +8°C) and stirred solution containing 3 mg of RSA per milliliter of 0.1 M sodium hydrogen carbonate (NaHCO_3). The molar ratio between the added anhydride and RSA was 60:1. The mixtures were stirred for 18 hours. The anhydride-RSA conjugates were purified from low-molecular-weight compounds (<30 000 Da), and the 0.1 M NaHCO_3 buffer was substituted by 0.1 M ammonium hydrogen carbonate (NH_4HCO_3) via ultrafiltration processing

Figure 1. Chemical structures of the organic acid anhydrides used in the experiments. (SA = succinic anhydride, DMSA = dimethylsuccinic anhydride, DESA = diethylsuccinic anhydride, MA = maleic anhydride, MMA = methylmaleic anhydride, *cis*-HHPA = *cis*-hexahydrophthalic anhydride, 4-MHHPA = 4-methylhexahydrophthalic anhydride, PA = phthalic anhydride, 4-MPA = 4-methylphthalic anhydride, TMA = trimellitic anhydride, 3,4,5,6-THPA = 3,4,5,6-tetrahydrophthalic anhydride, 1,2,3,6-THPA = 1,2,3,6-tetrahydrophthalic anhydride, 3,4-MTHPA = 3,4-methyltetrahydrophthalic anhydride, 4,4-MTHPA = 4,4-methyltetrahydrophthalic anhydride)

(Amicon Co, Denver, MA, USA) and stored at -20°C after lyophilization.

Determination of hapten density. The hapten density (HD) was determined for the conjugates by different methods. The "UV method" was a spectrophotometric one, according to Zeiss et al (11). In the "HCl method" the conjugates were hydrolyzed at 3 selected concentrations of hydrogen chloride (HCl) (0.1, 1, or 6 M), respectively. The samples were evaporated and then worked-up and analyzed by GC-MS according to Lindh & Jönsson (18). The hydrolysis conditions giving the highest yield were chosen for the determinations. The "TNBS method" was based on the titration of free amino groups by 2,4,6-trinitrobenzene sulfonic acid (TNBS), according to Snyder & Sobocinski (19).

Immunization

Free anhydride. Unconjugated, free anhydrides were dissolved in dioxane; then liquid paraffin was added to make a 0.2 M solution (the final concentration of dioxane was 3%). The rats were immunized via intradermal injection with 0.1 ml (in 2 portions of 0.05 ml) of the freshly prepared solutions. Each anhydride was injected into 7 animals. As a control, 7 rats were intradermally injected with liquid paraffin; the dose was 0.1 ml. Serum samples were collected 28 days after the immunization.

Conjugate. The procedures for the conjugates were the same for the free anhydrides, but with anhydrides conjugated to RSA. The dose was 1.4 mg of SA-RSA, or cis-HHPA-RSA, or MA-RSA, respectively, in 0.14 ml of 0.15 M sodium chloride (NaCl). Seven rats were intradermally injected with RSA as a control group, the dose was 1.4 mg.

Antibody determinations by enzyme-linked immunosorbent assay

Specific immunoglobulin E. The following steps were used for specific Ig E: (i) polystyrene microtiter plates (Nunc-Immuno Plate, Nunc, Kamstrup, Denmark) were coated by adding 100 μl /well with 0.015% (in phosphate-buffered saline) anhydride-RSA conjugate; (ii) uncoated protein-binding sites were blocked by adding 200 μl /well with 4% bovine serum albumin and the plates were stored with blocking solution at -18°C until use; (iii) sera taken from immunized BN rats were added to the plates at 100 μl /well in 2-fold dilutions, starting from 1:50, and incubated for 1 hour at room temperature; (iv) sheep anti-rat Ig E (ICN ImmunoBiologicals Inc, Costa Mesa, CA, USA) of dilution 1:3000 was added at 100 μl /well and incubated for 1 hour at room temperature; (v) alkaline phosphatase-conjugated rabbit anti-sheep Ig G (Fc Fragment; ICN Biomedicals) of dilution 1:5000 was added at 100 μl /well and incubated for 1 hour at room temperature [the

plates were washed after each addition with a Titertek Microplate Washer (Flow Laboratories, Rickmansworth, United Kingdom)]; and (vi) substrate (p-nitrophenyl phosphate, Sigma Chemical Co) of 0.1% in diethanolamine buffer was added at 100 μl /well. After 2 hours at room temperature, the wells were read at 405 nm by a filter photometer (Titertek Multiskan, Eflab Oy, Helsinki, Finland). All the samples were analyzed in triplicate, with RSA-coated wells as controls for nonspecific binding. The result of each sample was expressed as the value of optical density (OD). The titer was the highest dilution at which the corresponding OD value was greater than the mean OD value of control rat sera plus 3 standard deviations. If the "mean OD + 3SD" was less than 0.05, the latter value was taken as the limit.

Cross-inhibition tests. Antisera of the rats immunized with 4-MHHPA were pooled and diluted at 1:50 in phosphate-buffered saline. Various anhydride-RSA or anhydrides conjugated to guinea pig serum albumin (GPSA) were added to the sera at concentrations of 0, 0.0064, 0.032, 0.16, 0.8 and 4 mg/ml, and after incubation at 4°C for 20 hours, specific Ig E against 4-MHHPA-RSA was analyzed.

Specific immunoglobulin G. The analysis for Ig G was performed according to the method for specific Ig E, but alkaline phosphatase-conjugated goat anti-rat Ig G (working dilution 1:4000; H + L, Zymed Laboratories, Inc, San Francisco, CA, USA) was added directly in the fourth step.

Statistics

For comparisons of the distributions between different groups, the Mann-Whitney U-test was used. The Spearman rank correlation (rs) method was applied to investigate the correlation between 2 variables which can be expressed in a rank order. Statistical significance refers to $P \leq 0.05$ (2-tailed).

Results

The hapten density for all the conjugates ($N=14$) was analyzed by the TNBS method. It varied between 16 and 27 mol/mol for the different conjugates (table 1). For 6 of the conjugates, the molar absorbances were large enough to permit determinations also by the UV method. Four of these conjugates corresponded well with the results from the TNBS method, while MA-RSA and TMA-RSA showed a higher and lower hapten density, respectively. The hapten densities after the acidic hydrolysis were lower than those determined by the other 2 methods.

The titers of specific Ig E after the immunization of the rats with the free anhydrides varied from negative to 6400 (table 2). SA and 3,4,5,6-THPA did not give

positive results. Titers (median) below 1000 were obtained with DMSA, DESA, MA, MMA, 1,2,3,6-THPA, 3,4-MTHPA and 4,4-MTHPA, while titers above 1000 were obtained with cis-HHPA, 4-MHHPA, PA, 4-MPA, and TMA. The rats immunized with 4-MPA exhibited the highest titers, which were significantly different from the titers of all the other OAA. 4-MPA and DMSA also demonstrated significant differences in titers compared with their nonmethylated analogues PA and SA, respectively.

In the ELISA (enzyme-linked immunosorbent assay) inhibition tests with anti-4-MHHPA sera, the strongest inhibition was shown by 4-MHHPA-RSA at each concentration of the conjugate. For this anhydride a 50% inhibition occurred at a conjugate concentration of <0.0064 mg/ml in the antisera, and the inhibition was >95% when the conjugate concentration in the antisera was 0.16 mg/ml. In addition 4,4-MTHPA-RSA, 4-MHHPA-GPSA, and cis-HHPA-RSA showed inhibitions of >90% at the

highest concentration (4 mg/ml) employed. For the same anhydride (4-MHHPA, cis-HHPA, 4-MPA), the inhibition of the RSA conjugate was larger than that of the corresponding GPSA conjugate (figure 2).

The titers of specific Ig G varied from 200 to 6400 (table 2). As for Ig E, 4-MPA exhibited the highest titers ($P \leq 0.05$), and SA and 3,4,5,6-THPA had the lowest titers. There was a close correlation between the Ig E and Ig G titers for the various OAA ($r_s = 0.92$, $P = 0.0001$).

The tested conjugates also induced the formation of positive specific Ig E and Ig G. The titers of Ig E varied from 50 to 800, and those for specific Ig G varied from 200 to 1600 (table 3). cis-HHPA-RSA induced higher titers than either SA-RSA or MA-RSA.

The lack of correlation ($r_s = 0.3$, $P = 0.5$) between the Ig E titers and the reactivity of the anhydrides demonstrated by their hydrolysis rate constants according to Ebersson & Landström (20) is shown in figure 3.

Table 1. Hapten densities of the conjugates between different organic acid anhydrides and rat serum albumin at the molar ratio of 60:1 as determined by a spectrophotometric method (UV method), the analysis of the corresponding acids after acidic [hydrogen chloride (HCl) hydrolysis of the conjugates] (HCl method), and indirect analysis by the determination of free amino groups by 2,4,6-trinitrobenzene sulfonic acid (TNBS method). (NA = not analyzed)

Conjugate	Hapten density (mol / mol)			
	UV method	HCl method	HCl	TNBS method
SA-RSA (succinic anhydride-rat serum albumin)	NA	11	6	23
DMSA-RSA (dimethylsuccinic anhydride-rat serum albumin)	NA	7	6	22
DESA-RSA (diethylsuccinic anhydride-rat serum albumin)	NA	NA	..	23
MA-RSA (maleic anhydride-rat serum albumin)	48	11	0.1	23
MMA-RSA (methylmaleic anhydride-rat serum albumin)	28	7	0.1	22
cis-HHPA-RSA (cis-hexahydrophthalic anhydride-rat serum albumin)	NA	10	0.1	20
4-MHHPA-RSA (4-methylhexahydrophthalic anhydride-rat serum albumin)	NA	13	0.1	22
1,2,3,6-THPA-RSA (1,2,3,6-tetrahydrophthalic anhydride-rat serum albumin)	NA	11	0.1	24
3,4,5,6-THPA-RSA (3,4,5,6-tetrahydrophthalic anhydride-rat serum albumin)	21	10	0.1	24
3,4-MTHPA-RSA (3,4-methyltetrahydrophthalic anhydride-rat serum albumin)	NA	NA	..	16
4,4-MTHPA-RSA (4,4-methyltetrahydrophthalic anhydride-rat serum albumin)	NA	NA	..	25
PA-RSA (phthalic anhydride-rat serum albumin)	26	19	6	27
4-MPA-RSA (4-methylphthalic anhydride-rat serum albumin)	26	NA	..	27
TMA-RSA (trimellitic anhydride-rat serum albumin)	13	NA	..	22

Table 2. Titers of specific immunoglobulin (Ig) E and G by enzyme-linked immunosorbent assay of sera from rats (N=7) after intradermal immunization respectively with 0.1 ml of 0.2 M solutions of different organic acid anhydrides in liquid paraffin.

Anhydride	IgE titer		IgG titer	
	Median	Range	Median	Range
SA (succinic anhydride)	<50	<50	<50	<50
DMSA (dimethylsuccinic anhydride)	200	100-400	800	400-1600
DESA (diethylsuccinic anhydride)	800	400-800	1600	800-1600
MA (maleic anhydride)	800	800-3200	1600	1600-3200
MMA (methylmaleic anhydride)	200	100-200	400	200-800
cis-HHPA (cis-hexahydrophthalic anhydride)	1600	1600-3200	3200	1600-3200
4-MHHPA (4-methylhexahydrophthalic anhydride)	1600	800-1600	3200	1600-3200
1,2,3,6-THPA (1,2,3,6-tetrahydrophthalic anhydride)	800	200-1600	800	200-1600
3,4,5,6-THPA (3,4,5,6-tetrahydrophthalic anhydride)	<50	<50-50	<50	<50-50
3,4-MTHPA (3,4-methyltetrahydrophthalic anhydride)	800	400-800	1600	800-1600
4,4-MTHPA (4,4-methyltetrahydrophthalic anhydride)	800	800-3200	1600	800-3200
PA (phthalic anhydride)	3200	1600-3200	3200	1600-3200
4-MPA (4-methylphthalic anhydride)	3200	3200-6400	6400	3200-6400
TMA (trimellitic anhydride)	1600	800-6400	1600	800-3200

Figure 2. The enzyme-linked immunosorbent assay inhibition test. Pooled sera from the rats immunized with 4-methylhexahydrophthalic anhydride were incubated with the conjugates between different organic acid anhydrides (OAA) and rat serum albumin (RSA) or guinea pig serum albumin (GPSA) at concentrations of 4, 0.8, 0.16, 0.032, and 0.0064 mg/ml at 4°C for 20 hours; thereafter immunoglobulin E was analyzed from 4-MHHPA-RSA coated plates. The anhydride conjugates are listed from top to bottom in increasing order according to their inhibition. (SA = succinic anhydride, DMSA = dimethylsuccinic anhydride, MA = maleic anhydride, MMA = methylmaleic anhydride, cis-HHPA = cis-hexahydrophthalic anhydride, 4-MHHPA = 4-methylhexahydrophthalic anhydride, PA = phthalic anhydride, 4-MPA = 4-methylphthalic anhydride, TMA = trimellitic anhydride, 1,2,3,6-THPA = 1,2,3,6-tetrahydrophthalic anhydride, 4,4-MTHPA = 4,4-methyltetrahydrophthalic anhydride)

Table 3. Titers of specific immunoglobulin (Ig) E and G by enzyme-linked immunosorbent assay of sera from 3 groups of the rats (N=7) after intradermal immunization with 1.4 mg of different conjugates between organic acid anhydrides and rat serum albumin in saline.

Antigen	IgE titer		IgG titer	
	Median	Range	Median	Range
SA-RSA (succinic anhydride-rat serum albumin)	200	50-800	400	200-1600
MA-RSA (maleic anhydride-rat serum albumin)	200	100-400	400	200-1600
cis-HHPA-RSA (cis-hexahydrophthalic anhydride-rat serum albumin)	800	400-800	800	800-1600

Discussion

Positive specific Ig E and Ig G titers were obtained after intradermal immunization of rats with various free OAA. However, the magnitude of the induced titers varied. SA and 3,4,5,6-THPA failed to give detectable antibody production, while the highest specific Ig E titers were shown by cis-HHPA, 4-MHHPA, TMA, and especially PA and 4-MPA. A close correlation was exhibited between the Ig E and Ig G titers. Immunization with the corresponding RSA conjugates showed positive titers for SA, MA, and cis-HHPA.

Equimolar amounts of the anhydrides were used for the immunizations. It is of course possible that the effective amounts differed because of the different levels of stability of the anhydrides before injection. However, in guinea pigs, similar titers were obtained after immunization with 0.3 M suspensions and 0.2 M solutions, respectively (Zhang et al, unpublished observations). Thus the exact amount of anhydride does not seem to be critical at the administered dose of immunization. A 10-fold decrease in concentration for very reactive OAA before immunization may have a substantial effect. However, very

high titers were observed for the reactive MA in guinea pigs (15).

The antibody titers analyzed by ELISA may be dependent on the quality of the hapten conjugate used for the determinations. If the conjugates have a different hapten density, they may show different responses in the assays (21). However, there are indications that hapten densities in the range of 10–25 mol/mol are optimal and show a low variation in activity (21, 22). The conjugates in the present study had hapten densities of 16–27 mol/mol, as demonstrated by the TNBS method. These results were confirmed by the HCl method. Lower values were obtained by the HCl method. However, the hapten densities analyzed by the HCl method also fell in the range of 10–25, except for DMSA-RSA and MMA-RSA, both of which had a hapten density of 7. On the other hand, hapten densities as low as 6 have been shown to exhibit only minor reductions in antibody titers (22). Thus we believe that the conjugates in our work were within their optimal hapten density range.

To identify and produce the optimal conjugates for a large number of haptens is expensive and time-consuming. Thus it has been suggested that the total Ig E induced

by immunization may be used as an alternative indicator of the immunogenicity of a chemical (5). However, this possibility has to be tested further.

Ig E and Ig G are 2 different classes of anaphylactic antibodies in rats, both of which increase after active sensitization (23, 24). There was a close correlation between the Ig E and Ig G titers in this study. Both antibody classes showed similar patterns and levels of titers.

SA showed negative results, while a positive titer of Ig E was induced by MA. The present negative findings for SA, as compared with MA, can be explained by the flexibility of the succinic acid molecule and the fact that SA is an endogenous compound. In addition, MA may react by the double bond with thiol groups in proteins. However, the immunogenicity of MA is interesting, in light of the small size of the molecule. The antibody titers increased when SA was substituted with methyl groups (DMSA), and even more so when the substituents were ethyl groups (DESA). The titers increased still more when DESA was ring closed to the more rigid cis-HHPA. Further methylation to 4-MHHPA caused no additional increase in the titers. However, even higher titers were observed after immunization with the corresponding aromatic anhydrides PA and 4-MPA. When the methyl group in 4-MPA was replaced by a carboxyl group (TMA), the titers significantly decreased. Thus the chemical character of the substituent is important. The results of PA and TMA agree with the observed serum Ig E levels in mice after topical application of the free anhydrides (9). Substitution of MA by MMA and 3,4,5,6-THPA showed a different pattern of induced antibodies as compared with the effect caused by the corresponding structural changes of SA. A possible explanation may be the instability of the MMA and 3,4,5,6-THPA conjugates under in-vivo conditions (25). The isomerization of the double-bond position in 3,4,5,6-THPA to the 1,2,3,6-THPA isomer showed a significant positive effect on the Ig E titers. A corresponding effect is seen when the position of the methyl group in MTHPA is shifted from the 3- to the 4-position on the 6-carbon ring.

The specificity of the antibody readings was demonstrated by the low readings of the RSA controls on the ELISA microtiter plates, as well as the low readings for the control animals immunized with RSA. The specificity of the antibodies was further demonstrated by the ELISA inhibition tests of specific Ig E to 4-MHHPA. 4-MHHPA-RSA presented the highest inhibition. Other OAA showed different inhibitions, depending on their structural similarities with 4-MHHPA, as was demonstrated in an earlier work (26). Thus cis-HHPA-RSA and 4,4-MTHPA-RSA showed inhibitions close to that of 4-MHHPA-RSA. Corresponding inhibitory potentials were obtained for the different RSA and GPSA conjugates. For the same anhydrides, the RSA-conjugates exhibited higher inhibitions than those of the corresponding GPSA conjugates.

Figure 3. Correlation ($r_s=0.3$, $P=0.5$) between induced Ig E titers and the hydrolysis rate constants of succinic anhydride (SA), dimethylsuccinic anhydride (DMSA), diethylsuccinic anhydride (DESA), cis-hexahydrophthalic anhydride (cis-HHPA), maleic anhydride (MA), methylmaleic anhydride (MMA), and 3,4,5,6-tetrahydrophthalic anhydride (3,4,5,6-THPA) according to Eberson & Landström (20).

However, the differences were limited and therefore demonstrated a high hapten specificity of the antibodies.

OAA induce antibody formation by conjugation in vivo; the conjugates are recognized as "nonself" proteins by the immune system. Thus antibody formation after immunization may be influenced by the chemical reactivity of the anhydrides (7). However, no correlation was seen between the hydrolysis rate constants and the antibody titers in the present work, as was observed in a previous study of guinea pigs (15). Thus there is no simple correlation between chemical reactivity and sensitizing potential. The chemical reactivity of a low-molecular-weight allergen is only one of several parameters which may affect sensitization.

Whereas the results of immunization with the free anhydrides may reflect several other characteristics of the anhydrides, including solubility and reactivity, immunization with the corresponding serum-albumin conjugates mainly reflects the "nonself" recognition of the conjugate. Thus immunization with free anhydrides and corresponding protein conjugates, respectively, may give different information on the sensitizing potential of the anhydrides. Immunization with cis-HHPA-RSA, MA-RSA, and SA-RSA showed positive antibody formation. If a hapten density of about 25 mol/mol is assumed, the amount of free anhydride bound to the protein carrier used for immunization is about 1/30 of the doses of free anhydrides used for immunization. Thus immunization with the conjugate appears to be more efficient than with free anhydride. This difference may simply reflect the amount of free anhydride which reacts with proteins after injection. Interestingly, free SA did not induce detectable antibody levels, but SA-RSA did. A low sensitizing potential of SA has also been demonstrated by the lack of response to

bronchial challenge in SA-sensitized guinea pigs (Zhang et al, unpublished data). Rats immunized with SA or MA bound to the RSA carrier induced similar titers but differed markedly after immunization with the unconjugated ones. This finding may indicate an effect of both chemico-physical characteristics and the epitopic structures on the sensitizing potential.

A correlation ($r_s=0.63$, $P<0.05$) can be seen between the rat Ig E titers in the present study and the Ig G₁ titers after the immunization of guinea pigs with the same anhydrides in a previous study (15) (table 4). Passive cutaneous anaphylaxis tests of specific guinea pig Ig E suggested an enhanced effect on Ig E by methyl group substitution. However, in the present study, methyl group substitution in PA and SA resulted in increased titers, while in cis-HHPA no effect was found and in MA there was a decreasing effect. Thus there may be an animal species difference.

A close correlation was observed between specific Ig G₁ titers and lung resistance in guinea pigs sensitized with cis-HHPA (27). The relationship between specific Ig E and the airway response in rats was not evaluated by us in this study. However, a relationship between an increased dose of antigen, an increased Ig E titer, and enhanced airway resistance has been demonstrated earlier (28).

There are indications that chemical allergens that cause respiratory tract sensitization in humans also induce sensitization in experimental animals after intradermal or topical immunization (5). However, there is no conclusive evidence that animal models can predict the sensitizing potential of chemicals in humans (1, 29). There is a reasonable agreement between the results from the present rat model and epidemiologic studies of exposed workers. Hence studies on exposed workers have shown a high prevalence of positive specific Ig E in workers exposed to MTHPA (14), TMA (30), cis-HHPA, and 4-MHHPA (31), while PA has shown lower Ig E levels (13). There are, to our knowledge, no reports of specific Ig E in

humans to SA, DMSA, DESA, MA, MMA, 1,2,3,6-THPA, 3,4,5,6-THPA, and 4-MPA. However, this lack of evidence may be due to a lack of investigations on these chemicals or due to the fact that they have little use in industry.

In conclusion, the magnitude of the specific Ig E titers in rats after intradermal immunization showed interesting relationships with the chemical structures of the various OAA. Importantly, the results agreed well with those from a corresponding study of Ig G₁ in guinea pigs, and with findings from exposed workers. Thus the present model may be a valuable tool for predicting the sensitizing potential of low-molecular-weight compounds. Furthermore, the antibody specificity to the haptens demonstrated by the inhibition tests and the variation in antibody titers after immunization make the OAA an interesting model for studies on quantitative structure-activity relationships.

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References

1. Briatico-Vangosa G, Braun CLJ, Cookman G, Hofman T, Kimber I, Loveless SE, et al. Respiratory allergy: hazard identification and risk assessment. *Fundam Appl Toxicol* 1994;23:145–58.
2. Karol MH. Animal models of occupational asthma. *Eur Respir J* 1994;7:555–68.
3. Agius RM, Nee J, McGovern B, Roberson A. Structure-activity hypothesis in occupational asthma caused by low molecular weight substances. *Ann Occup Hyg* 1991;35:129–37.
4. Karol MH, Graham C, Gealy R, Macina OT, Sussman N, Rosenkranz HS. Structure-activity relationships and computer-assisted analysis of respiratory sensitization potential. *Toxicol Lett* 1996;86:187–91.
5. Kimber I, Hilton J, Basketter D, Dearman RJ. Predictive testing for respiratory sensitization in the mouse. *Toxicol Lett* 1996;86:193–8.
6. Pauluhn J. Predictive testing for respiratory sensitization. *Toxicol Lett* 1996;86:177–85.
7. Wass U, Belin L. An in vitro method for predicting sensitizing properties of inhaled chemicals. *Scand J Work Environ Health* 1990;16:208–14.

Table 4. Rat serum immunoglobulin (Ig) E titers (median, N=7) and guinea pig serum IgG₁ titers (median) [Welinder et al (15)] analyzed by enzyme-linked immunosorbent assay after intradermal immunization with free organic acid anhydrides.

Antigen	Rat IgE titer (median)	Guinea pig IgG ₁ titer (median)
SA (succinic anhydride)	<50	300
MA (maleic anhydride)	800	51 200
MMA (methylmaleic anhydride)	200	6400
cis-HHPA (cis-hexahydrophthalic anhydride)	1600	12 800
4-MHHPA (4-methylhexahydrophthalic anhydride)	1600	12 800
1,2,3,6-THPA (1,2,3,6-tetrahydrophthalic anhydride)	800	6400
3,4,5,6-THPA (3,4,5,6-tetrahydrophthalic anhydride)	<50	800
4,4-MTHPA (4,4-methyltetrahydrophthalic anhydride)	800	102 400
3,4-MTHPA (3,4-methyltetrahydrophthalic anhydride)	800	25 600
PA (phthalic anhydride)	3200	51 200
4-MPA (4-methylphthalic anhydride)	3200	51 200
TMA (trimellitic anhydride)	1600	12 800

8. Sarlo K, Clark ED. A tier approach for evaluating the respiratory allergenicity of low molecular weight chemical. *Fund Appl Toxicol* 1992;18:107—14.
9. Dearman RJ, Basketter DA, Kimber I. Variable effects of chemical allergens on serum IgE concentration in mice. Preliminary evaluation of a novel approach to the identification of respiratory sensitizers. *J Appl Toxicol* 1992;12:317—23.
10. Dearman RJ, Basketter DA, Kimber I. Differential cytokine production following chronic exposure of mice to chemical respiratory and contact allergens. *Immunology* 1995;86:545—50.
11. Zeiss CR, Patterson R, Pruzansky JJ, Miller MM, Rosenberg M, Levitz D. Trimellitic anhydride-induced airway syndromes: clinical and immunologic studies. *J Allergy Clin Immunol* 1977;60:96—103.
12. Howe W, Venables KM, Topping MD, Dally MB, Hawkins R, Law JS, et al. Tetrachlorophthalic anhydride asthma: evidence for specific IgE antibody. *J Allergy Clin Immunol* 1983;71:5—11.
13. Nielsen J, Welinder H, Schütz A, Skerfving S. Specific serum antibodies against phthalic anhydride in occupationally exposed subjects. *J Allergy Clin Immunol* 1988;82:126—33.
14. Welinder H, Nielsen J, Gustavsson C, Bensryd I. Specific antibodies to methyltetrahydrophthalic anhydride in exposed workers. *Clin Exp Allergy* 1990;20:639—45.
15. Welinder H, Zhang XD, Gustavsson C, Björk B, Skerfving S. Structure-activity relationships of organic acid anhydrides as antigen in an animal model. *Toxicology* 1995;103:127—36.
16. Tada T, Okumura K, Platteau B, Beckers A, Bazin H. Half-lives of two types of rat homocytotropic antibodies in circulation and in the skin. *Int Arch Allergy Appl Immunol* 1975;48:116—31.
17. Sorkness R, Blythe S, Lemanske RF JR. Pulmonary antigen challenge in rats passively sensitized with a monoclonal IgE antibody induces immediate but not late changes in airway mechanics. *Am Rev Respir Dis* 1988;138:1152—6.
18. Lindh CH, Jönsson BAG. Determination of hexahydrophthalic acid and methylhexahydrophthalic acid in plasma after derivatization with pentafluorobenzyl esters using gas chromatography and mass spectrometric detection. *J Chromatography* 1997;691:331—9.
19. Snyder SL, Sabocinski PZ. An improved 2,4,6-trinitrobenzene sulphonic acid method for the determination of amines. *Anal Biochem* 1975;64:284—8.
20. Eberson L, Landström L. Studies on cyclic anhydrides. IV. Rate constants for the hydrolysis of some cyclic anhydrides exhibiting ring strain. *Acta Chem Scand* 1972;26:239—49.
21. Topping MD, Venables KM, Luczynska CM, Howe W, Newman Taylor AJ. Specificity of the human IgE response to inhaled acid anhydrides. *J Allergy Clin Immunol* 1987;77:834—42.
22. Welinder H, Nielsen J. Immunologic tests of specific antibodies to organic acid anhydrides. *Allergy* 1991;46:601—9.
23. Binaghi RA, Perrudet-Badoux A. Additive activity of IgE and IgG anaphylactic antibodies in rats. *Ann Immunol Paris* 1976;127:49—56.
24. Yeadon M, Price R. Induction of calcium-independent nitric oxide synthase by allergen challenge in sensitized rat lung in vivo. *Br J Pharmacol* 1995;116:2545—6.
25. Palacian E, Gonzales MP, Pineiro M, Hernandez F. Dicarboxylic acid anhydrides as dissociating agents of protein-containing structures. *Mol Cell Biochem* 1990;97:101—11.
26. Zhang X-D, Lötvall J, Skerfving S, Welinder H. Antibody specificity to chemical structures of organic acid anhydrides studied by in-vitro and in-vivo methods. *Toxicology* 1997;118:223—32.
27. Zhang X-D, Lötvall Y, Arahawa H, Welinder H, Skerfving S. Relationship between IgG₁ levels and airway responses in guinea pigs actively and passively sensitized to hexahydrophthalic anhydride. *Allergy* 1998;53:20—7.
28. Watanabe A, Hayashi H. Allergen-induced biphasic bronchoconstriction in rats. *Int Arch Allergy Appl Immunol* 1990;11:71—7.
29. Selgrade MJK, Zeiss CR, Karol MH, Sarlo K, Kimber I, Tepper JS, et al. Workshop on status of test methods for assessing potential of chemicals to induce respiratory allergic reactions. *Inhalation Toxicol* 1994;6:303—19.
30. Zeiss CR, Mitchell JH, Van-Peenen PF, Harris J, Levitz D. A twelve-year clinical and immunologic evaluation of workers involved in the manufacture of trimellitic anhydride (TMA). *Allergy Proc* 1990;11:71—7.
31. Welinder HE, Jönsson BAG, Nielsen JE, Ottosson HE, Gustavsson CA. Exposure-response relationships in the formation of specific antibodies to hexahydrophthalic anhydride in exposed workers. *Scand J Work Environ Health* 1994;20:459—65.

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