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International Immunopharmacology 2 (2002) 239–248

International Immunopharmacology

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Review

Immunotoxicology of organic acid anhydrides (OAAs)

Xing Dong Zhang, Paul D. Siegel, Daniel M. Lewis*

Analytical Services Branch, Health Effects Laboratory Division, NIOSH, Morgantown, WV 26505, USA

Abstract

Organic acid anhydrides (OAAs) have considerable economic importance due to their extensive use in the production of alkyd, epoxy, and polyester resins. Occupational exposure to OAAs has been associated with a variety of health effects, which may be classified into two major categories of direct toxicity/irritant and hypersensitivity. The hypersensitivity diseases associated with OAA exposure are thought to be related to the reactivity of these chemicals and in particular their ability to form protein conjugates that may be recognized as neo-antigens by the immune system. This review will present a brief discussion of the basic chemistry of these compounds and the environmental and biological monitoring methods used for exposure measurements. The clinical syndromes associated with exposure to these compounds will be discussed along with factors that may affect disease susceptibility. Finally, animal models that have been developed to examine the mechanisms of disease will be discussed. Published by Elsevier Science B.V.

Keywords: Organic acid anhydrides; Occupational asthma; Occupational lung disease; Animal models

1. Introduction

Organic acid anhydrides (OAAs) are reactive chemicals that have considerable economic importance due to their extensive use in the production of alkyd, epoxy and polyester resins. As a class, OAAs can produce a wide spectrum of health effects that have been generally classified into two major categories of direct toxicity/irritancy and immunologically mediated hypersensitivity [1]. This review will focus on the hypersensitivity reactions. The respiratory tract is considered the major site of hypersensitivity reactions, but skin has also been reported as a target organ. Three OAA hypersensitivity syndromes have been described. The first is allergic asthma, clinically defined by the symptoms of coughing, wheezing and dyspnea, and thought to be mediated by IgE (allergic) antibodies. OAAs can also produce flu-like symptoms (cough, dyspnea, fever, chills and myalgia) 4–12 h after exposure and this reaction called the late respiratory systemic syndrome (LRSS) is possibly mediated by IgG antibodies and/or cell mediated immune reactions similar to hypersensitivity pneumonitis type reactions. The third syndrome, the pulmonary disease–anemia (PDA) syndrome, may also present with flu-like symptoms, but is characterized by dyspnea, pulmonary infiltration and hemorrhage, hemolysis and anemia. The underlying mechanism of PDA is less clear, but it is thought to have an immunologic basis as well.

Abbreviations: HHPA, hexahydrophthalic anhydride; ID, intradermal; IL, interleukin; LRSS, late respiratory systemic syndrome; MHHPA, methylhexahydrophthalic anhydride; MTHPA, methyltetrahydrophthalic anhydride; OAA, organic acid anhydride; PDA, pulmonary disease anemia; PA, phthalic anhydride; Th2, T helper type 2 lymphocytes; TMA, trimellitic anhydride; TWA, time-weighted average.

1567-5769/02/$ - see front matter. Published by Elsevier Science B.V.
PII: S1567-5769(01)00176-X

This review of OAAs is roughly divided in three main topic categories: chemistry/environmental and biological monitoring, occupational health aspects and animal studies. The general chemical characteristics of OAAs is central to understanding their ability to induce disease. Methods used to quantify exposure through environmental and biological monitoring are also discussed as these are the measures used to relate dose to disease. The occupational health section reviews the spectrum of clinical syndromes that have been associated with OAA exposure and factors that contribute to OAA disease and susceptibility. The final section covers animal studies and how they have contributed to the understanding of mechanisms from exposure to the development of clinical disease.

2. Chemistry of OAAs

OAAs are used in many different chemical products. Some of the most economically important OAAs and their uses are listed in Table 1. The structures of several common OAAs reported to induce asthma are shown in Fig. 1. The toxicity and immunogenicity of OAAs are probably related to their chemical reactivity. Anhydrides can react with functional groups found on biological molecules such as hydroxyl, amine and thiol groups giving rise to conjugate formation. The protein–OAA conjugates may be immunogenic with the OAA acting as a hapten. The most important reaction in the haptenization of proteins is thought to be the ammonolysis reaction between OAA and a protein amino group forming a stable amide product. The extent of modification of other nucleophilic functional groups on a protein depends on both the amino acid and the particular OAA, as acylation and deacylation rates vary with both these factors and pH under aqueous conditions [2]. Although the mechanism of aspirin hypersensitivity remains controversial, it had been hypothesized that aspirin hypersensitivity may be mediated through *S*-acylation of cysteine by the aspirin metabolite *O*-acetylsalicylic anhydride [3]. Dannan et al. [3] suggested that the anhydride would initially react with a sulfhydryl protein group due to its greater nucleophilicity and the low number of reactive amines at physiological pH. Intramolecular transfer of the acyl group to the amine would follow, forming a thermodynamically stable amide product. The property of reversible *S*-acylation can be generalized to most, but not all OAAs. Maleic and methylmaleic anhydride can irreversibly acylate thiols and amines in proteins and peptides through a Michael addition type of reaction across the β, γ C−C double bond [2]. Thus, haptenization of proteins can proceed through several different chemical pathways leading to the generation of immunogenic products.

3. Environmental and biological exposure monitoring

Individual OAAs can vary greatly in vapor pressure. Exposure to a specific OAA can occur as a vapor/gas, a particulate or both simultaneously and this will dictate the industrial hygiene method used for exposure

Table 1
Organic acid anhydrides and select products/uses

OAA	Products/uses
Hexahydrophthalic anhydride	Insecticides, rust preventatives
Maleic anhydride	Hard contact lenses, hair spray, controlled release pharmaceutical tablets, styrene plastic, paper sizing, fiber glass reinforcement, concrete curing agents, electrodeposition polymer car coating
Phthalic anhydride	Dyes, pesticides, pharmaceuticals, cosmetics, produce isatoic anhydride used to make saccharin, flame retardants, rubber vulcanization retarder, fast-curing appliance finish
Pyromellitic anhydride	Thermoplastic coating used in electrical components insulation, laminates, enamels and adhesives
Succinic anhydride	Starch modifier
Trimellitic anydride	Luminescent dyes for plastic, wall and floor coverings; thermoplastics used in electrical connectors, bushings and jet engine parts

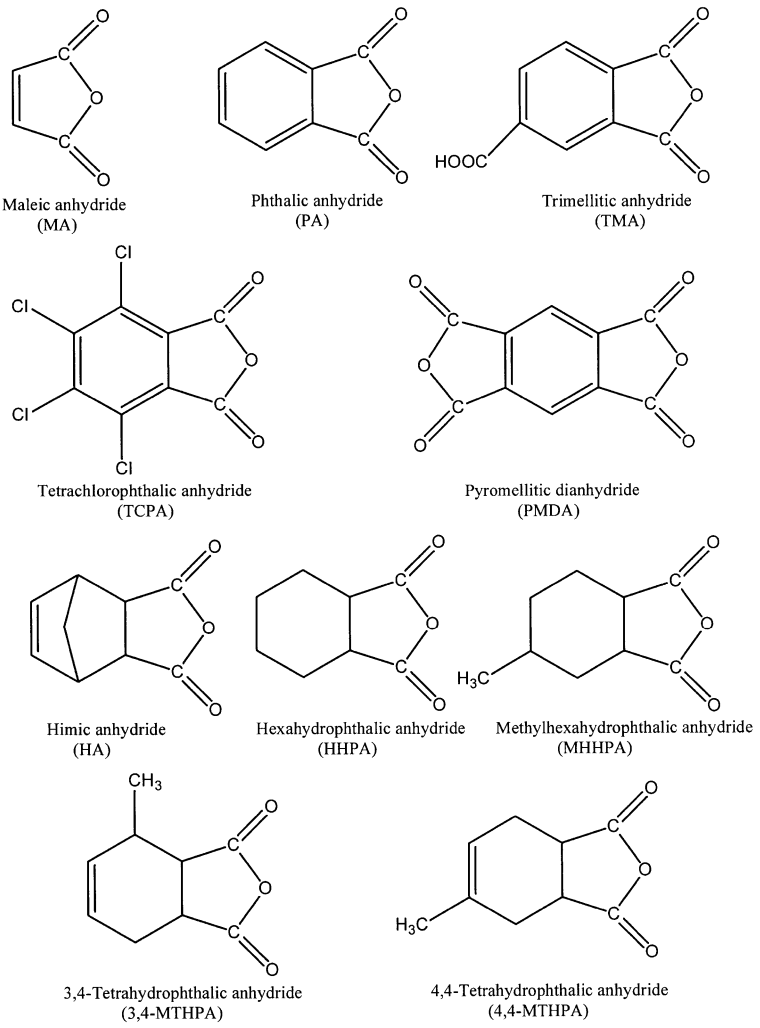

Fig. 1. Chemical structures of 10 organic acid anhydrides used in industry.

assessment. Particulates are usually sampled gravimetrically by trapping the chemical onto a filter. Environments that contain gases can be sampled by use of sorbent media or impingers alone or in combination with a filter trap. Sorbent materials that have been used include Amberlite XAD-2 [4,5], Tenax [6] and silica gel [7]. Sample preparation consists of extraction/desorption followed by derivitization to a more stable/more volatile form and analysis by gas (GC) or liquid chromatography (HPLC) with flame ionization (FID), electron capture (ECD), mass spectral (MS) or ultraviolet (UV) detectors. Gases can also be analyzed in the reactive form by GC-FID. These methods allow for the detection of very low concentrations of anhydrides with limits of detection reported as low as 1 μg/m^3 [7]. Direct analysis of hexahydrophthalic anhydride (HHPA) in air by fourier transformed infrared spectroscopy (FTIR) has also been reported [8]. FTIR can be useful to quickly survey a site for areas of high exposure, but is less precise (CV = 22% at 150 μg/m^3) and sensitive (limit of detection = 120 μg/m^3) than other methods. A more complete discussion of methods for determination of OAAs in air can be found in reviews (Parts 1 and 2) by Jonsson et al. [9,10].

Either IgG- or IgE-specific antibodies or OAA metabolites/reaction products can be used as bio-

markers of exposure. The presence of OAA-specific antibodies in a worker's serum suggests that the worker has been exposed previously to OAA or exposed to an agent that generates a cross-reactive antibody response. It takes several weeks to mount a measurable antibody response to an exposure and, thus, the specific antibody titer does not reflect a very recent exposure. Absence of specific antibody does not prove no or low exposure, as antibody responsiveness is variable in exposed individuals. The specific antibody response and its relevance to disease will be discussed in more detail later in this review.

Studies have been reported examining the utility of metabolites/reaction products of several different OAAs as biomarkers of exposure including phthalic anhydride (PA) [11,12], HHPA [5,13–16], methylhexahydrophthalic anhydride (MHHPA) [16] and methyltetrahydrophthalic anhydride (MTHPA) [17]. OAAs are subject to hydrolysis to the corresponding carboxylic acid, which may either occur in the body spontaneously or enzymatically. The specific, free carboxylic acid has been measured in pre- and post-shift urine and plasma. These carboxylic acids are excreted as free (unbound) acids. Some discrepancies exist with respect to biological half-lives ($t_{1/2}$). Pfaffli et al. [15] reported that the HHPA acid $t_{1/2}$ was 14 h. Jonsson and Skerfving [14] determined the HHPA acid $t_{1/2}$ to be approximately 2 h from volunteers exposed to controlled laboratory test atmospheres, while the $t_{1/2}$ was 5 h in workers exposed in an industrial setting [13]. In general, most studies show a very high correlation ($r \geq 0.9$) between end of the shift, specific free carboxylic acid urine and/or plasma concentrations and time-weighted air concentrations of that work shift. Jonsson et al. [13] also examined potential percutaneous absorption of HHPA as a contributor to the corresponding acid in the urine and found very low dermal uptake suggesting that dermal exposure probably contributed little to the urinary and plasma metabolites.

OAA–hemoglobin adducts have also been examined from red blood cells of exposed workers as a biological measure of exposure [16]. HHPA and MHHPA were found bound mainly to lysine residues in the hemoglobin. A good correlation was found between exposure, urinary metabolites and hemoglobin adducts. In this study, it was assumed that the workers OAA exposure was relatively constant and the body burden had reached a steady state allowing to correlate a single-day exposure and hemoglobin adduct level. The biological life-span for hemoglobin protein is approximately 4 months and adduct elimination from individuals who had left the job or had taken a 33-day vacation closely fit that predicted from normal hemoglobin elimination curves.

4. Occupational health aspects

Occupational exposure to OAAs has been associated with a variety of adverse health effects. In general, the toxic effects can be placed into two broad categories, irritant effects and respiratory hypersensitivity [1]. Direct irritant effects of OAAs on the skin and mucosal membranes are well documented and appear to be related to relatively high exposures. Such exposures were more common when OAAs were first used in industrial processes; for example, aerosol exposures of PA up to 40 mg/m^3 were reported from manufacturing facilities in the 1950s [1]. In recent years, hypersensitivity reactions have become the most significant occupational health concern, but irritant effects are still reported [18]. The current exposure limit in the United States of 0.04 mg/m^3 for TMA is for an 8-h time-weighted average (TWA). Intermittent high exposures can lead to irritant reactions, while the TWA remains in accepted ranges [1].

The hypersensitivity reactions induced by OAAs can result in at least three distinct clinical syndromes: (1) allergic rhinitis and/or asthma mediated by IgE antibodies; (2) late respiratory systemic syndrome (LRSS) possibly mediated by IgG antibodies and/or cell mediated immune reactions similar to hypersensitivity pneumonitis type reactions; and (3) pulmonary disease anemia (PDA), a form of acute chemical mediated pulmonary edema and hemorrhage that may be mediated by antibodies [18–20]. All three of these syndromes have been associated with TMA exposure, the most studied of the OAAs, but other anhydrides have also been implicated. The overarching process is that OAAs function as haptens; i.e. they react with proteins to form neo-antigens and induce an immune response. The clinical syndrome that results from exposure to OAAs is a reflection of the nature of the immune response (i.e., IgE, IgG and/or cell mediated responses) to the altered protein, the protein

or tissue modified by the reaction with OAA, the route and dose of the exposure, and the susceptibility of the exposed individual. Each of these points will be considered separately.

The mechanisms by which the immune response is directed to be an IgE, IgG or cellular response are not fully understood. In man, repeated mucosal exposure to low doses of an antigen is associated with IgE antibody responses that are thought to be driven by T helper 2 (Th2) secreted cytokines. Thus, occupational exposures to aerosols containing OAA, either as fine particulates or fumes, are thought to be the most significant risk factor for allergic (IgE) sensitization [21]. The potential role of dermal exposures leading to sensitization in man can not be excluded, and IgE mediated contact urticaria has been reported [22]. Kanerva et al. [23] also reported that a worker exposed to MHHPA developed allergic contact dermatitis (type IV allergy), allergic rhinitis and immediate contact skin reaction (type I).

Factors which influence the development of IgG antibodies and increase the risk for LRSS are less clear. Exposure level is clearly a factor [19] and there is significant overlap with exposure levels associated with IgG and IgE responses, but in general exposures associated with LRSS tend to be higher [24].

The immune factors that contribute to the hemorrhagic or PDA disease processes are ill defined. Early reports describing a hemorrhagic pulmonary disease associated with exposure to TMA fumes suggested that this was a direct toxic reaction based on the relatively high exposures, high attack rates, and the absence of antibodies [25]. Other studies reported high levels of specific antibodies to the OAA to which the subjects were exposed [26]. The presence of specific antibody is a marker of exposure and does not prove that an immune reaction was the underlying mechanism for PDA [1]. Regardless of the mechanism, it has been suggested that any OAA may cause pulmonary hemorrhage and anemia [20], and that high exposure levels are associated with the syndrome [18].

The reactivity of OAAs suggests that they could easily conjugate with multiple proteins resulting in the development of a variety of novel antigenic epitopes. This has implications with respect to the sensitivity and specificity of immunoassays designed to detect antibodies to OAAs [27]. In addition, the ability of OAA protein adducts to form on epithelial or red cell membranes may be important in the immunopathology on LRSS and PDA. Direct proof of this concept has not demonstrated in man, but animal studies have shown that TMA may be localized on airway epithelial cells and conjugated with proteins other than albumin [28].

In addition to exposure, other possible risk factors for sensitization to OAAs include smoking, atopic status and genetic factors. Mapp et al. [29], in their review of occupational asthma, included the acid anhydrides in a list of agents that appear to induce asthma through an IgE-mediated mechanism. The risk factors reported for the development of allergic asthma due to OAAs should, thus, be similar to those for environmental or occupational asthma to proteins. Atopy is a significant risk factor for protein mediated allergic asthmas [30]. Gautrin et al. [31] reported a trend for TMA asthmatics to be atopic, although the trend did not reach statistical significance. In contrast, Barker et al. [21] reported atopy to be a risk factor. Differing study design and definitions of sensitization may have lead to the different findings in these studies. Atopy may alter the dose response relationship between antigen (OAA) and sensitization. It is possible that sensitization to the occupational allergen in atopic individuals may occur more quickly or at lower exposure doses. Laboratory animal caretakers, for example, are at risk of developing occupational allergic diseases, and atopic status has been clearly defined as a risk factor for sensitization, especially within the first two years of employment [31]. Thus, one possible explanation for the lack of identifying atopy as a risk factor for sensitization to OAAs may be a lack of discrimination between exposure level or temporal relation between initial exposure and onset of disease.

The presence of specific IgE antibodies, as stated previously, is a biomarker of exposure but not diagnostic of asthma. Assays for IgE antibodies to OAAs have better sensitivity and specificity than do many assays for other low molecular weight asthmatogens, but the positive predictive value of these tests range from 19% to 83% [32] suggesting that other factors may be significant in disease expression. In a careful review of risk factors in a large cohort (401 subjects) of OAA-exposed workers, both exposure and smoking were identified as risk factors for sensitization

(skin prick test positive), but only exposure was a risk factor for respiratory symptoms [21]. The same investigators reported that sensitization to OAA was a risk factor for bronchial hyperresponsiveness (BHR) to histamine in OAA-exposed workers, but that exposure was not [33]. Finally, an association between IgE antibodies to OAA and a specific HLA-DR phenotype has been reported [34], but others have failed to confirm this association [35].

5. Animal models for OAA-induced asthma/lung injury

Guinea pig, mouse and rat models have been employed to investigate specific aspects of sensitization, and immunological and toxic responses associated with OAA exposure. These models have endeavored to mimic the phenotypic expression of OAA asthma reported from clinical studies and case reports, and provide data to refute or support potential mechanisms. OAA asthma manifests a Th2 phenotype which is characterized by both early and late phase airway obstruction and eosinophilic inflammation, with or without pulmonary hemorrhage. Specific IgE and IgG antibodies to OAA are commonly reported in OAA asthmatics. Animal studies have been successful in replicating this phenotype and have examined questions concerning route of sensitization, development of immunological and physiological responses, tolerance, OAA cross-reactivity, toxicokinetics and potential utility of biomarkers. There is considerable variation in experimental study designs, especially with respect to exposure routes. Traditionally, the respiratory tract has been considered the major route of OAA exposure leading to sensitization in the work place. However, concern has been recently raised over the potential of dermal OAA exposure as a route of sensitization.

5.1. Airway exposure to OAAs

It has been reported that Balb/c mice develop specific IgE and IgG antibodies within 2–3 weeks after inhalation exposure to TMA (1 h/day for 3 days at the concentration of 5 mg/m^3) [36]. Sprague–Dawley rats and guinea pigs also have been reported to produce specific antibodies after inhalation exposure to TMA or PA dust [37–40]. Immediate airway responses have been assessed in guinea pig models with varying results. Airway responses were not elicited from TMA sensitized guinea pig using a TMA protein conjugate [37], airway responses were induced with PA protein conjugate but not free PA [40]. Down-regulation of IgE, pulmonary responses and immunological tolerance in rats after repeated inhalation exposures to TMA have also been found [38,39]. Further work, however, is needed to investigate these phenomena as it may provide important information toward understanding the mechanisms leading to asthma and potential interventions to alleviate/prevent asthmatic disease.

Lung hemorrhage and injury observed in humans after exposure to TMA have also been investigated in the rat and guinea pig models. It was hypothesized that the formation of TMA immune complexes and subsequent activation of the complement system upon exposure plays a role in initiating lung inflammation and hemorrhage [41–44]. Inhalation of PA can induce lung hemorrhage in a guinea pig model [40]. Leach et al. [42] reported TMA exposure in rats produced a concentration-related increase in hemorrhagic foci, alveolar macrophage accumulation, alveolar hemorrhage, pneumonitis, IgG antibody and complement activation. They stated that the timing and nature of the lesions, along with the presence of lung IgG and complement, were consistent with other hypersensitivity pneumonitis models. Zeiss et al. [28] examined the localization of TMA and the relationship of antibody to lung injury/hemorrhage in the Sprague–Dawley rats. TMA was localized to alveolar and bronchial cells and trace amounts of haptenized protein were found in lavage fluid. An increase in TMA-specific antibodies in lymph node secreting cells, lung lavage and serum paralleled the increase in lung injury with continued exposure.

5.2. Exposure via nonairway routes

Intradermal (ID) injection of OAAs was first examined in the 1940s. It was found that guinea pigs developed specific antibodies after ID injection with free PA in olive oil or corn oil [45]. In the 1980s and 1990s, studies were reported using ID injection of free TMA or HHPA into guinea pigs to induce specific IgG1 and IgE antibodies [46,47]. Various exposure routes to HHPA, including subcutaneous, intraperitoneal and ID

injection, have been examined in guinea pigs. Intradermal injection produced higher titers of specific antibodies than other routes of sensitization [47]. The reasons for the greater efficacy of the ID route may be due to the rich protein nature of the dermis facilitating OAA haptenization over hydrolysis. Alternatively, antigen presenting cells, i.e., Langerhans' cells, are rich in the epidermis. OAAs for ID injection are prepared as suspensions in plant (olive or corn) oil or liquid paraffin, or solubilized in solutions containing organic solvents (acetone or dioxane). Since all anhydrides are subject to hydrolysis in water to their nonreactive, corresponding acids, water must be avoided when preparing the suspension/solution.

Airway responses have also been elicited by inhaling free TMA, TMA–protein or HHPA–protein conjugates in animals sensitized by ID injection [46–50]. IgG1 is the main cytotrophic antibody in the guinea pig and it is involved in the induction of allergic airway responses in that species [51]. The role of IgE antibodies in allergic/asthmatic responses in the guinea pig is not clear and study of these antibodies is limited by lack of specific antibodies to guinea pig IgE. IgE antibodies have been shown to play a role in the induction of mediator release in guinea pigs [52]. Analysis of specific IgE in a guinea pig can be performed using the passive cutaneous anaphylaxis (PCA) test 7 days after passive transfer of test sera into the skin of a naive guinea pig. However, the PCA test is less sensitive and quantitative than ELISA [53]. To study the role of specific IgE in guinea pig models in OAA allergic lung diseases, work is needed to develop specific immunochemical reagents for the guinea pig.

The guinea pig has been traditionally used because of the reactivity of its airways. Respiratory rate for conscious guinea pigs and lung resistance for anesthetized guinea pigs have been used as indices of immediate airway responses. Lung resistance, a good index of an asthma-like reaction, presently can only be measured in anesthetized animals. This is a limitation when studying late airway responses, which occur 5–8 h after challenge. Respiratory rate changes, that are often used as an index of immediate airway response, were not found to be significantly different between experimental and control guinea pigs during the late phase response after challenge [54].

A noninvasive method of sensitization to OAAs has also been developed in rodent models where OAAs dissolved in 4:1 acetone/olive oil are applied topically to an area of the skin [55–58]. With this method, animals produce higher levels of interleukin (IL)-4, IL-5, IL-10 cytokines secreted by Th2 cells and higher levels of IgE after application of OAAs than after the application of contact chemical allergens such as 2,4-dinitrochlorobenzene. These findings support the theory that human OAA asthma is mediated through a Th2 mechanism that may involve specific IgE.

Arts et al. [57] compared Brown Norway and Wistar rats following sensitization (skin painting) and inhalation challenge to TMA/acetone. They reported an immediate sharp decrease in respiratory rate during challenge followed by an increased respiratory rate and decreased tidal volume at 24 and 48 h post challenge. The Brown Norway rats displayed higher IgE responses, as well as histopathological and respiratory responses, than the Wistar strain. The histopathology in the sensitized, inhalation challenged Brown Norway rats observed were associated with increased IgE and resembled allergic asthma. Interestingly, similar, but less frequent and severe inflammation was noted in nonsensitized, inhalation challenged rats. Sensitization-dependent granulomatous inflammation, consistent with those reported for hypersensitivity pneumonitis and hemorrhages at higher inhalation challenges, were also observed.

All previously reported OAA dermal sensitization studies used test material made soluble in a vehicle to dose the skin. Recent studies in our laboratory have examined the ability of dry, powdered TMA to induce sensitization upon dermal exposure since a potential for dermal exposure to TMA powder exists in industry. Dry TMA powder administered topically to the skin of Brown Norway rats produced dose-dependent, specific IgE and IgG production [59]. In addition, sensitization by application of dry TMA powder to the skin produced immediate and late phase airway responses following inhalation challenge with TMA dust (data not published). Airway responses were monitored continuously for 24 h in unrestrained, conscious animals. Airway obstruction was reflected as an increase in enhanced pause (Penh). The dry TMA powder sensitization model may have more occupational health relevance than other models employing solvents or injection techniques.

5.3. Structure–activity relationships

In guinea pig and rat models, immunogenicity of 15 different OAAs were evaluated by ID injection of the OAAs. The findings suggested that the ring structure, position of double bonds and methyl group substitution altered the immunogenicity as assessed by antibody response [47,60,61]. Such studies may lead to models capable of predicting the allergic potential of similar low molecular weight chemicals.

5.4. Pathology of OAA asthma in animals

In guinea pigs and rats challenged by inhalation of PA or TMA following sensitization by a nonairway route, the airway submucosa is infiltrated with eosinophils and lymphocytes. Airways are occluded and damage of the bronchial epithelium evident. This pathological sequela is similar to that observed in other animal models of allergic asthma [57,62,63]. The histopathological response of OAA sensitized, challenged animals, as noted previously, can be a mixed response with hypersensitivity pneumonitis-type lesions and hemorrhage [57]. The spectrum of histopathological lesions observed in animal models of OAA is consistent with the OAA-associated clinical diseases reported in man.

6. Conclusion

In summary, OAAs are important chemicals of commerce which, due to their chemical reactivity, can react with or modify biological molecules leading to a variety of airway and systemic diseases. Toxic- and sensitization-dependent OAA diseases include LRSS, PDA and asthma. Studies suggest that sensitizing exposures may occur through both the respiratory tract and skin. The dose, route and duration of exposure are all important in determining the type and extent of physiologic and/or pathologic outcome.

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