

The Direct Peptide Reactivity Assay: Selectivity of Chemical Respiratory Allergens

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It is well known that some chemicals are capable of causing allergic diseases of the skin and respiratory tract. Commonly, though not exclusively, chemical allergens are associated with the selective development of skin or respiratory sensitization. The reason for this divergence is unclear, although it is hypothesized that the nature of interactions between the chemical hapten and proteins is influential. The direct peptide reactivity assay (DPRA) has been developed as a screen for the identification of skin-sensitizing chemicals, and here we describe the use of this method to explore whether differences exist between skin and respiratory allergens with respect to their peptide-binding properties. Known skin and respiratory sensitizers were reacted with synthetic peptides containing either lysine (Lys) or cysteine (Cys) for 24 h. The samples were analyzed by HPLC/UV, and the loss of peptide from the reaction mixture was expressed as the percent depletion compared with the control. The potential for preferential reactivity was evaluated by comparing the ratio of Lys to Cys depletion (Lys:Cys ratio). The results demonstrate that the majority of respiratory allergens are reactive in the DPRA, and that in contrast to most skin-sensitizing chemicals, preferentially react with the Lys peptide. These data suggest that skin and respiratory chemical allergens can result in different protein conjugates, which may in turn influence the quality of induced immune responses. Overall, these investigations reveal that the DPRA has considerable potential to be incorporated into tiered testing approaches for the identification and characterization of chemical respiratory allergens.

Key Words: chemical allergens; hazard identification; skin sensitization; respiratory sensitization; peptide reactivity.

The most common manifestation of immunotoxicity in humans is skin sensitization resulting in allergic contact dermatitis (ACD), with several thousand chemicals identified as being potential sensitizers (De Groot, 2008). By comparison, chemical respiratory allergy is less common and there are far fewer (< 100) known causative agents (EU, 2008). However, a diagnosis of respiratory sensitization, characterized by asthma and

rhinitis, is associated with high levels of morbidity and significant socioeconomic costs (Bernstein *et al.*, 2006; Vandenplas *et al.*, 2003). Due to the important health consequences, chemical respiratory allergy remains an area of considerable importance for toxicology and occupational health.

The prospective identification of chemical respiratory allergens has presented a significant challenge for toxicologists, and there are currently no validated or generally accepted methods available for hazard identification (Isola *et al.*, 2008; Kimber *et al.*, 2007). In contrast, predictive methods for the identification of skin sensitizers have been available for some time, including guinea pig tests (Buehler, 1965; Magnusson and Kligman, 1969) and the local lymph node assay (LLNA) (Kimber *et al.*, 2002, 2011). The development of these assays has been facilitated by an increasing understanding of the chemical and biological mechanisms of skin sensitization (Kimber *et al.*, 2011).

The lack of validated assays for the identification of chemical respiratory allergens is in large part a reflection of uncertainty about the relevant immunobiological mechanisms and the events that result in the development of sensitization of the respiratory tract to chemicals. Although chemical respiratory allergy is associated with the development of selective T-helper (Th)2 cell responses (promoting immediate-type allergic hypersensitivity), there remains uncertainty, and some controversy, about a mandatory universal requirement for IgE antibody (Bernstein *et al.*, 2002; Kimber and Dearman, 2005; Kimber *et al.*, 2007). A further complication is that the term “asthmagen” has been coined to describe all materials that cause the symptoms of asthma/rhinitis, regardless of whether this is achieved via an immunological mechanism indicative of allergic sensitization. This distinction is important as respiratory sensitizers (but not other astmagens) will result in chronic adverse health effects due to immunological priming.

Despite resulting in different forms of allergic disease, skin and respiratory sensitizers share many common characteristics.

The chemicals themselves are of low molecular weight and capable of inducing immune activation only after covalent modification of proteins (Weltzien *et al.*, 1996). Thus, sensitizing chemicals are naturally electrophilic, or can be converted to electrophilic species in target tissues, allowing them to react with nucleophilic amino acids within proteins (Smith and Hotchkiss, 2001). Consistent with this, surveys of the electrophilic chemistry of respiratory allergens and the development of structure-activity relationship models have demonstrated that similar reaction mechanisms exist to those observed for skin sensitizers (Cunningham *et al.*, 2005; Enoch *et al.*, 2010).

There are significant, and increasing, societal, scientific, and economic pressures to develop alternative methods to replace animal testing (EU, 2003). To this end, a number of investigators have developed screening methods based on peptide and/or protein reactivity to identify potential skin sensitizers, including the direct peptide reactivity assay (DPRA) (reviewed in Gerberick *et al.*, 2008). The DPRA utilizes two peptides containing either lysine (Lys) or cysteine (Cys) as surrogate nucleophiles; the test material is reacted with either peptide and the subsequent depletion of peptide from the reaction mixture is used as a quantitative measure of reactivity (Gerberick *et al.*, 2004). Overall, increased peptide depletion in the DPRA has been shown to display a general association with relative sensitizing potency as measured by the LLNA (Gerberick *et al.*, 2007). Although the development of these reactivity assays were founded on experience with skin-sensitizing chemicals, it has been proposed that a similar approach could be applied to the prospective identification of respiratory allergens (Lalko *et al.*, 2011).

The purpose of the investigations described here was, therefore, twofold. The first objective was to evaluate whether chemical respiratory allergens could be identified in the DPRA. To this end, the reactivity of 20 low molecular weight respiratory sensitizers was characterized for Cys and Lys in the DPRA. These materials are representative of the most common classes of industrial chemicals (diisocyanates, anhydrides, and reactive dyes) and electrophilic reaction mechanisms associated with respiratory allergy (Fig. 1; Chan-Yeung and Malo, 1994; Dearman *et al.*, 1997; Enoch *et al.*, 2010; Health and Safety Executive [HSE], 2001). The second goal was to determine if respiratory allergens display different characteristics of amino acid selectivity when compared with skin-sensitizing chemicals. For this purpose, the relative depletion of the Lys and Cys peptides was compared between the two classes of materials. The results suggest that an important mechanistic distinction may exist between skin and respiratory allergens with respect to peptide and protein binding.

MATERIALS AND METHODS

Test materials. The test materials used in these studies included chemical respiratory and skin sensitizers. All chemicals were dispensed and experiments prepared in a fume cupboard with gloves for skin protection.

The following chemicals (identified also by CAS number) were purchased from Aldrich Chemical Company (Milwaukee, WI): 1-butanol, 99.5% (71-36-3); cinnamaldehyde, 99% (14371-10-9); cyanuric chloride, 99.0% (108-77-0); diethyl maleate, 97% (141-05-9); diethyl phthalate, 99.5% (84-66-2); ethyleneglycol dimethacrylate (EGDM), 98% (97-90-5); hexahydrophthalic anhydride, 95.0% (85-42-7); 2-phenylpropionaldehyde, 98% (93-53-8); tetrachlorophthalic anhydride, 96.0% (117-08-8). Other chemicals were purchased from Sigma Chemical Company (St Louis, MO): basic blue 99, 50.0% (68123-13-7); *p*-benzoquinone, 98% (106-51-4); black GR reactive dye, 55.0% (17095-24-8); 2,4-dinitrochlorobenzene (DNCB), 99% (97-00-7); ethylenediamine, 99.0% (107-15-3); glutaraldehyde, 50.0% (111-30-8); hexamethylene diisocyanate, 99.0% (822-06-0); lactic acid, 85% (50-21-5); orange 3R reactive dye, 55.0% (12225-83-1); phthalic anhydride, 99.0% (85-44-9); piperazine, 99.0% (110-85-0); reactive yellow, 50.0% (10343-58-5). The following chemicals were purchased from TCI America (Portland, OR): diphenylmethane diisocyanate, 97.0% (101-68-8); toluene 2,4-diisocyanate, 98.0% (584-84-9); toluene 2,6-diisocyanate, 98.0% (91-08-7); 2,4,6-toluene diisocyanate (TDI), 99.0% (2647-62-5); trimellitic anhydride, 98.0% (552-30-7). 4-Allylanisole, 98% (140-67-0) and maleic anhydride, 99.0% (108-31-6) were purchased from Fluka Chemical Company (Milwaukee, WI). Chlorhexidine, 98.6% (55-56-1) was purchased from MP Biomedicals (Solon, OH). Chloramine-T, 95.0% (127-65-1) was purchased from Pfaltz & Bauer (Waterbury, CT).

Ammonium acetate, ammonium hydroxide, sodium phosphate monobasic, and sodium phosphate dibasic for use in the preparation of buffer solutions were purchased from J.T. Baker (Phillipsburg, NJ). HPLC grade acetonitrile (> 99.9%) was purchased from EMD (Gibbstown, NJ). HPLC grade trifluoroacetic acid (TFA, ≥ 99%) was obtained from Sigma-Aldrich (St Louis, MO). Ultrapure MilliQ water was prepared by filtration through a Milli-Q Academic Q10 with a Q Guard 2 pack and a point of use 0.22-μm Millipak 40 filter (Millipore, Billerica, MA).

DPRA. The DPRA was conducted according to the method of Gerberick *et al.* (2007). Heptapeptides containing Lys (Ac-RFAACAA-COOH) or Cys (Ac-RFAAKAA-COOH) were prepared by RS Synthesis (Louisville, KY). The peptides were purified to > 90% by HPLC and molecular weight confirmation was conducted via flow injection mass spectrometry with electrospray ionization in the positive mode. Peptide stock solutions were prepared to a final concentration of 0.667mM in 100mM ammonium acetate buffer (pH of 10.2) for the Lys peptide and in 100mM phosphate buffer (pH of 7.5) for the Cys peptide. Test chemical stock solutions were prepared to a concentration of 100mM in acetonitrile (adjusted for purity of the test compound). All stock solutions were prepared within 2h of use. Each reaction was prepared in triplicate. The Cys peptide reaction containing 0.5mM of the peptide and 5mM of the test chemical, representing a 1:10M ratio, was prepared by combining 750 μl of the Cys peptide stock solution, 200 μl acetonitrile, and 50 μl test chemical stock solution in HPLC glass autosampler vials (National Scientific, Rockwood, TN). The Lys peptide reaction containing 0.5mM of the peptide and 25mM of the test chemical, representing a 1:50M ratio, was prepared by combining 750 μl of the Lys peptide stock solution and 250 μl test chemical stock solution. Control samples were prepared by combining 750 μl of peptide solution with 250 μl of acetonitrile. Calibration standards were prepared for each peptide at concentrations of 0.0156, 0.0313, 0.0625, 0.125, 0.25, and 0.5mM in the appropriate buffer with acetonitrile. The vials were capped, gently vortexed, and allowed to incubate in the HPLC autosampler (in the dark) at 25°C for 24h.

Following incubation, the unreacted peptide was quantified by reverse-phase HPLC with UV detection. The chromatographic system comprised a Waters 2695 Alliance fitted with a Zorbax SB-C18 column (3.5 μm, 100 × 2.1 nm) (Agilent Technologies, Wilmington, DE). The stationary phase was preceded by a SecurityGuard cartridge system containing a C18 guard cartridge (2.0 × 4.0nm) (Phenomenex, Torrance, CA). UV detection was made by a Waters 996 PDA detector using an external linear calibration curve. Samples were processed via the auto sampler; 10 μl aliquots were injected onto the column that was maintained at 30°C. Separation was achieved using a 0.35 ml/min flow rate with a gradient of mobile phase A (0.1% vol/vol TFA in water) and mobile phase B (0.085% vol/vol TFA in acetonitrile) over a 20-min period. The

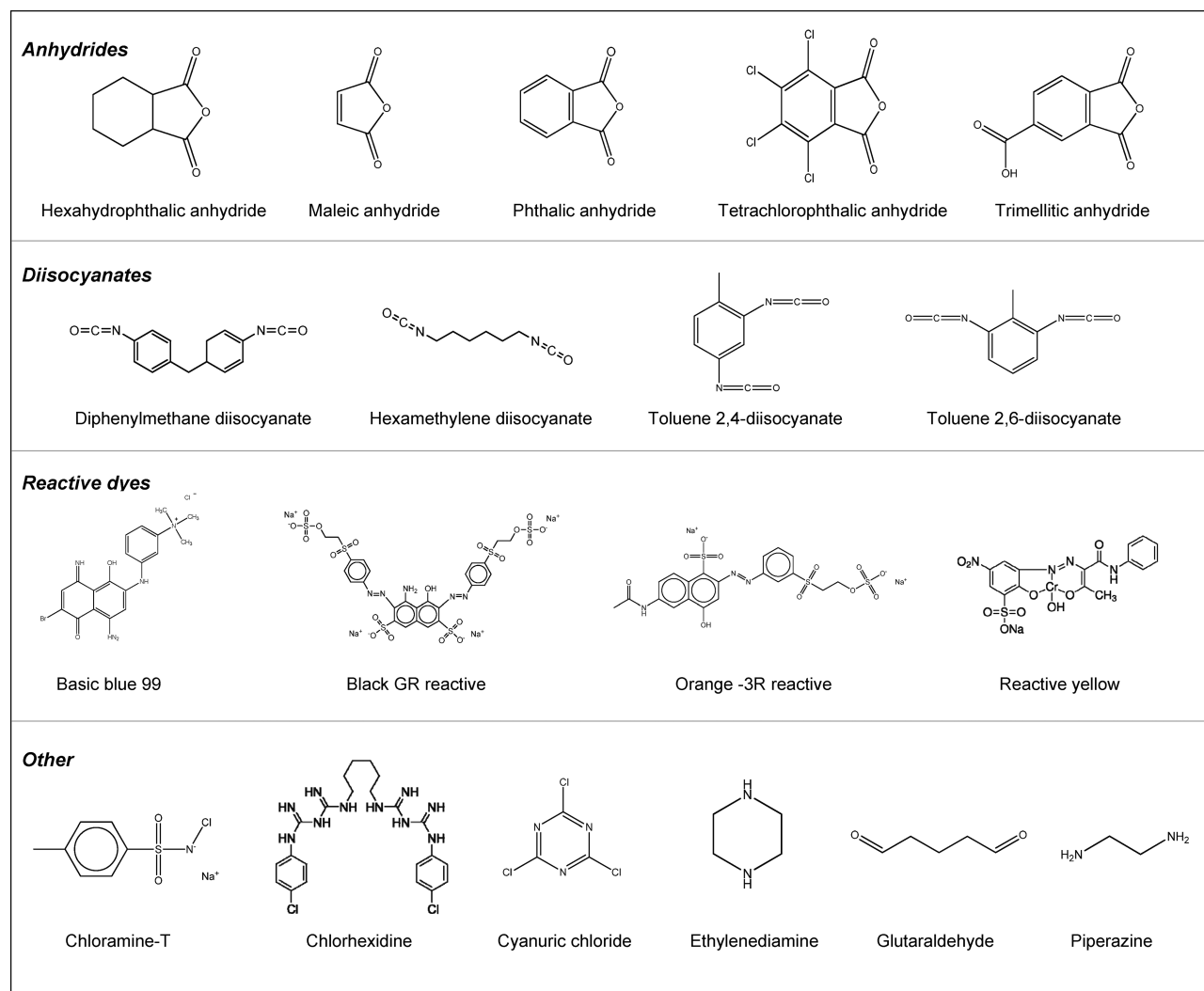


FIG. 1. The chemical structures of the respiratory allergens evaluated in the DPRA are presented. These materials are representative of common material classes and structures identified as occupational respiratory allergens (Chan-Yeung and Malo, 1994; Dearman *et al.*, 1997; HSE, 2001).

gradient was adjusted from 90% A/10% B to 10% A/90% B during the initial 13 min. The gradient was then rapidly returned to 90% A/10% B over 30 s for the remainder of the separation. The UV spectrum was collected from 210 to 400 nm to permit verification of the peak identity.

The data were collected and evaluated utilizing Empower Pro 2 software (Build 2154, Waters Corporation, Leederville, WA) running on the Microsoft XP (service pack 3) operating system. The concentration of peptide was determined in each reaction from absorbance at 220 nm. The appropriate peak was integrated yielding the peak area corresponding to the individual unreacted peptide in the test reactions, controls, and standards. A linear calibration curve was calculated based on the peptide concentration standards. The calibration curve was accepted as valid if the correlation coefficient (R^2) was > 0.990 . The peptide reactivity was then quantified as a percent depletion based on the decrease in unreacted peptide in the test sample relative to the average concentration in the controls.

Data analyses. The reproducibility of the peptide reactivity assay was evaluated for a subset ($n = 10$) of the results published by Gerberick *et al.* (2007). The relationship between the measured and published data was compared by linear regression, calculated along with the 95% confidence interval

(CI) for a best fit. Additionally, the positive control data obtained for cinnamic aldehyde were collated from 32 consecutive runs of the standard peptide reactivity assay. These data were best fitted to a normal distribution. From this distribution the mean, SD, and low (2.5%; 5%)/high (95%; 97.5%) percentiles were calculated. The data were evaluated using a *t*-test, where a *p* value of < 0.05 was considered to be statistically significant.

The peptide depletion values measured for the chemical respiratory allergens were compared with reactivity measurements for a series of skin sensitizers. In order to evaluate the distribution of reactivity to the Lys and Cys peptides by material class, the individual allergens were classified according to the extent that they depleted each peptide. The classifications were based on intervals of 10% depletion, i.e., those causing 0–10% depletion, those causing 11–20% depletion, etc. Cumulative percentage distributions were prepared, based on these classifications, on a “greater than” basis with each interval representing the percent of test chemicals that resulted in depletion values greater than the lower bound of the given interval. For example, the $> 0\%$ interval represents the percentage of all test chemicals that exhibited any reactivity to the peptide, the $> 10\%$ interval represents the percentage of test chemicals resulting in depletion of $> 10\%$, etc. Preferential reactivity was evaluated at the individual chemical level by calculating the ratio of Lys to Cys depletion (Lys:Cys ratio)

based on the mean peptide depletion for each test material. The Lys:Cys ratios were transformed to their log equivalent for evaluation. The data were evaluated using a Mann-Whitney test, where a p value of < 0.05 was considered to be statistically significant. All analyses were performed using GraphPad Prism 5 (La Jolla, CA).

RESULTS

Reproducibility of the DPRA

In order to establish the reproducibility of the DPRA and to confirm that comparisons with published datasets were appropriate, a subset of 10 materials examined by Gerberick *et al.* (2007) were selected and evaluated. As judged by the LLNA, these materials represented the full range of skin sensitization potential reported in the published dataset and included nonsensitizers (lactic acid, 1-butanol, diethyl phthalate), weak sensitizers (EGDM), moderate sensitizers (2-phenylpropionaldehyde, diethyl maleate, cinnamic aldehyde), and strong/extreme sensitizers (DNCB, *p*-benzoquinone) (Gerberick *et al.*, 2005). The Lys and Cys peptide depletion values obtained for these chemicals in the current study are shown in comparison with the previously published data in Figure 2. The relationship between the two datasets was evaluated by linear regression. Overall, the assay exhibited a high level of reproducibility (Lys, $R^2 = 0.97$; Cys, $R^2 = 0.85$), particularly for the Lys peptide. Consistent with the published dataset, each of the materials was correctly and reproducibly identified as either a sensitizer or nonsensitizer when utilizing the classification tree proposed previously by Gerberick *et al.* (2007). For example, diethyl

phthalate, lactic acid, and 1-butanol failed to cause marked depletion of either the Lys or Cys peptide resulting in a classification of minimally reactive (nonsensitizing). In contrast, the strong skin sensitizers DNCB and *p*-benzoquinone were correctly identified as highly reactive having resulted in marked depletion, approaching 100%, of at least one peptide.

During the course of the present investigations, the reaction of cinnamic aldehyde with the Lys- and Cys-containing peptides served as a positive control. These data provided the opportunity to evaluate both the variability of the DPRA and the amino acid selectivity over time for a single chemical. The control data were collated from 32 consecutive runs and fitted to a normal distribution (Fig. 3). The mean $\pm$ SD depletion of Lys by cinnamic aldehyde was $51.1\% \pm 6.3$ and for Cys it was $74.5\% \pm 6.6$. These differences are statistically significant ($p < 0.001$) and demonstrate that cinnamic aldehyde is more effective at depleting Cys when compared with Lys. To further characterize the variability, the low (2.5 and 5%) and high (95 and 97.5%) percentiles were calculated for the depletion of each peptide. For Lys, the low percentiles were 31.0%/32.0% depletion and the high percentiles were 69.0%/70.1% depletion. The low and high percentiles for Cys were 55.9%/56.9% depletion and 93.1%/94.1% depletion, respectively. Based on these lower and upper limits, the distributions of the Lys and Cys depletion data displayed a modest degree of overlap in the 50–70% range. This region of overlap represents a statistical possibility that cinnamic aldehyde could result in a greater depletion of Lys when compared with Cys. However, the data indicate that this does not occur in practice, and that experimental variability in the DPRA affects the reactivity of both peptides to a similar

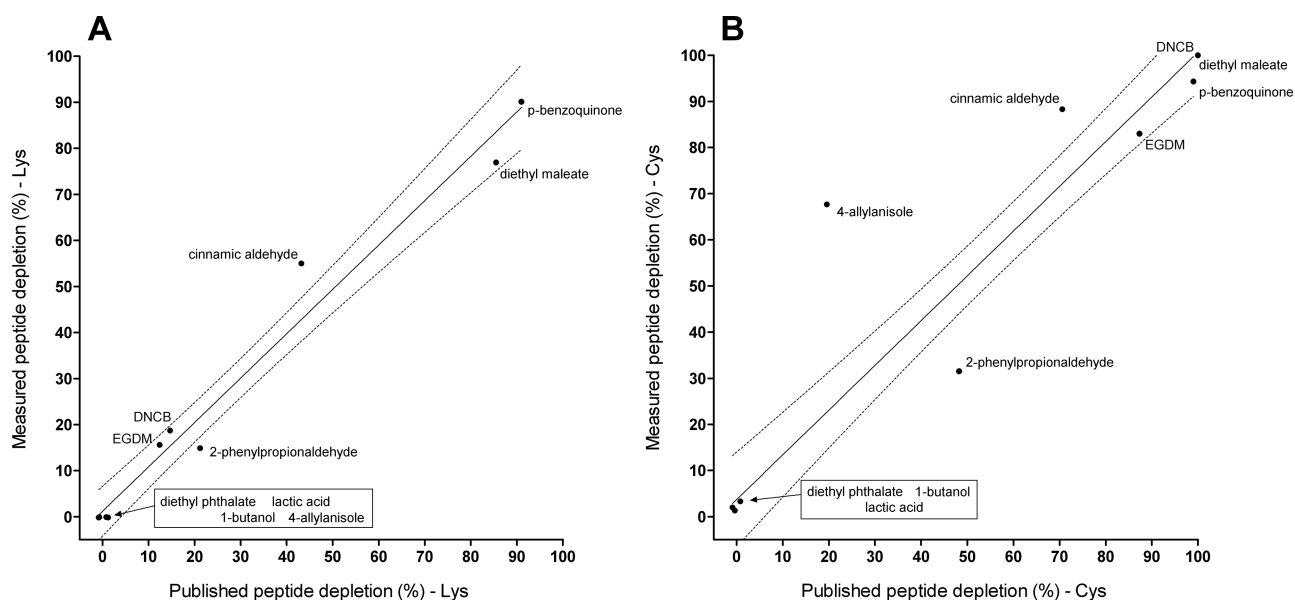


FIG. 2. The correlation between peptide depletion data reported by Gerberick *et al.* (2007) and that measured in this study conducted on a subset of the published results is presented. The assay was conducted according to the published protocol with (A) Lys reactivity being evaluated at a peptide: test material ratio of 1:50 at a pH of 10.2 and (B) Cys reactivity being evaluated at a ratio of 1:10 at a pH of 7.5. The relationship between the measured and published data was evaluated by linear regression (—; Lys, $R^2 = 0.97$; Cys, $R^2 = 0.85$), and the results are displayed graphically above with the 95% CI for a best fit (---).

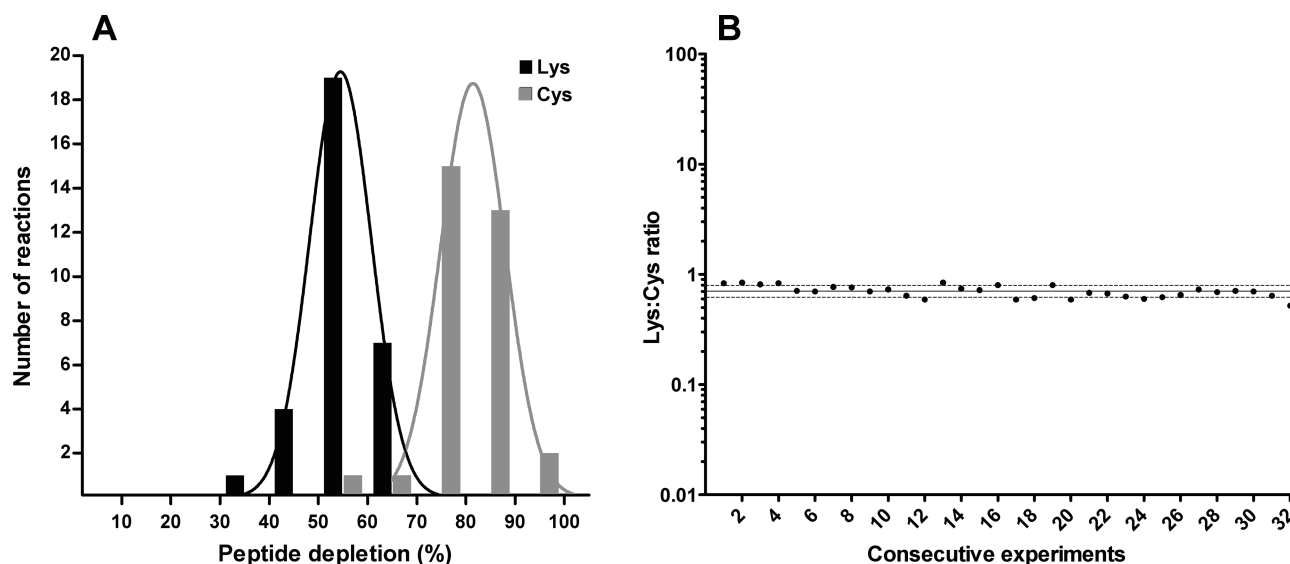


FIG. 3. Positive control data for the reaction of cinnamic aldehyde with a Lys- and Cys-containing peptide in the DPRA. Over a 1-year period, 32 consecutive experimental runs were completed with Lys and Cys reactivity being evaluated concurrently. (A) The individual depletion measurements for Lys and Cys were sorted based on peptide depletion intervals of 10%. A normal distribution was best fitted to the data (Lys, $R^2 = 0.99$; Cys, $R^2 = 0.98$). (B) The ratio of Lys to Cys depletion (Lys:Cys ratio, ●) was calculated for each individual experiment. This value was taken as a measure of the relative depletion of each peptide. The mean (—) $\pm$ SD (---) of the Lys:Cys ratio was 0.70 ± 0.08 .

degree. For example, despite the overlap in the distributions (comprised only 2 of 32 experiments), the depletion of the Cys peptide by cinnamic aldehyde was always observed to be greater than Lys on a within run basis. This preferential reactivity is illustrated in Figure 3, which shows the relative reactivity of cinnamic aldehyde toward the Lys and Cys peptides over time. The relative reactivity was defined by calculating the Lys:Cys ratio and was found to be stable and reproducible. The mean $\pm$ SD of the Lys:Cys ratio over 32 consecutive runs was 0.70 ± 0.08 . The low (2.5 and 5%) and high (95 and 97.5%) percentiles for the Lys:Cys ratio were calculated to be $0.52/0.56$ and $0.84/0.84$, respectively.

The results in Figs. 2 and 3 demonstrate that the DPRA is reproducible over time and provides a consistent method for measuring differences in selective reactivity to Lys and Cys between chemicals.

Chemical Respiratory Sensitizers: Peptide Depletion Measurements

The peptide depletion results for all respiratory sensitizers when reacted with either the Lys- or Cys-containing peptide are displayed in Table 1. Seventeen of the 20 respiratory allergens were observed to yield depletion measurements of above 10% for one or both of the peptides, in many cases by a significant margin. Ethylenediamine and piperazine were observed to deplete the Lys and Cys peptide at very low levels ($\leq 3.3\%$). Chlorhexidine failed to react with either peptide. Based on previous experience, depletion of less than 10% is typically associated with nonsensitizing chemicals or, in some cases, pre/prohaptens (Gerberick *et al.*, 2007).

Comparative Distribution of Peptide Depletion Data for Chemical Respiratory and Skin Sensitizers

Peptide depletion measurements for a series of skin-sensitizing chemicals, obtained under the standard experimental conditions of the DPRA, were previously published by Gerberick *et al.* (2007). Those data, together with results obtained with the skin sensitizers tested here, have been used here as the basis for comparisons between skin and respiratory allergens. The dataset ($n = 48$) comprised a wide range of chemical classes, potencies, and reactivity known to exist among skin sensitizers. Thirty-nine of the 48 skin sensitizers were reported to have some ability to deplete both the Lys and Cys peptides.

In order to determine if respiratory and skin sensitizers react differently to Lys and Cys, the overall distribution of depletion measurements were compared within and between the two classes. These distributions are presented in Figure 4. Due to the size difference in the dataset (respiratory allergens, $n = 20$; skin allergens, $n = 48$) the cumulative percentage distributions were used for comparison. These distributions were prepared on a “greater than” basis, where each interval represents the percentage of test chemicals that resulted in depletion values greater than the lower value of the interval. For the respiratory allergens, broadly similar distributions were obtained for both the Cys- and Lys-containing peptides and no statistically significant differences were observed. The general trend for these distributions was linear, demonstrating that the respiratory allergens exhibited the full spectrum of reactivity including very low to very high reactors to both peptides. For example, in both the Lys and Cys distributions, the $> 0\%$ depletion interval included 90% of the respiratory

TABLE 1
Peptide Depletion Measurements for 20 Respiratory Allergens in the DPRA

Test material	Peptide depletion (%) ^a			
	Lys		Cys	
	Average (n = 3)	SD	Average (n = 3)	SD
<i>Lys and Cys reactive</i>				
Basic blue 99	10.5	4.6	47.3	4.2
Black GR reactive dye	99.5	0.5	77.3	2.7
Chloramine-T	98.9	0.6	99.5	0.2
Cyanuric chloride	99.3	0.6	74.1	0.3
Diphenylmethane diisocyanate	25.1	3.7	83.5	2.6
Glutaraldehyde	79.6	4.0	35.5	3.4
Hexahydrophthalic anhydride	86.3	1.2	8.1	1.2
Hexamethylene diisocyanate	35.0	3.0	100.0	15.1
Maleic anhydride	93.0	1.0	54.0	0.8
Orange 3R reactive dye	97.3	3.5	93.5	3.1
Phthalic anhydride	94.0	1.0	10.9	3.5
Reactive yellow	79.3	1.7	82.1	0.8
Tetrachlorophthalic anhydride	84.3	4.9	2.3	0.5
Toluene 2,4-diisocyanate	23.0	1.6	54.5	3.2
Toluene 2,6-diisocyanate	26.3	4.2	87.4	5.8
Toluene 2,4/2,6-diisocyanate	18.5	0.7	15.9	1.8
Trimellitic anhydride	95.3	0.5	8.9	3.6
<i>Minimally/nonreactive</i>				
Chlorhexidine	-1.0	7.4	-2.2	0.8
Ethylenediamine	3.3	2.1	0.0	0.3
Piperazine	-2.0	8.0	1.6	4.0

^aThe reactions consisted of 0.5mM of peptide and either 5mM or 25mM of test material for reactions with Cys or Lys, respectively. The pH of the reactions was 7.5 (100mM phosphate buffer) for Cys and 10.2 (100mM ammonium acetate buffer) for Lys. All samples were prepared at a final reaction volume of 1 ml, and conducted in triplicate. Following 24-h incubation under ambient conditions, the reactions were analyzed by HPLC/UV and the loss of peptide from the reaction mixture was expressed as the average percent depletion ($\pm$ SD, $n = 3$) compared with the control.

allergens tested and the > 90% interval for Lys included 35% and for Cys 15% of the tested materials. This is in contrast to the distributions obtained for the skin sensitizers, which as a class exhibited a significantly greater ($p < 0.05$) depletion of the Cys- compared with the Lys-containing peptide. The general trend for the distribution obtained with the Cys peptide was linear with a large proportion of the skin sensitizers observed to result in high levels of depletion. For the Cys peptide, the > 0% depletion interval included 98% of the skin sensitizers tested and the > 90% interval included 42% of the test materials. The distribution obtained with the Lys peptide was observed to be skewed towards lower depletion levels. The > 0% depletion interval for Lys includes 81% of the test materials; however, the > 10% interval includes only 46% of the skin sensitizers (a similar level observed in the Cys distribution at the > 90% depletion interval).

Interestingly, when comparisons between classes of chemical allergen were made, the respiratory allergens were observed to result in greater depletion ($p < 0.01$) of the Lys peptide in comparison with the skin sensitizers at each level. For example, 55% of the respiratory allergens depleted the Lys peptide by > 50%, whereas only 13% of the skin sensitizers depleted the peptide at that same level. The opposite was observed when comparing the depletion of the Cys peptide by the two classes

of allergens. Overall, the skin-sensitizing chemicals resulted in greater depletion of the Cys peptide in comparison with the respiratory sensitizers, although the difference in the distributions did not reach statistical significance. In this case, for example, 63% of the skin and 50% of the respiratory sensitizers depleted the Cys-containing peptide by > 50%.

Ratio of Lys to Cys Peptide Depletion for Both Chemical Respiratory and Skin Sensitizers

Given the apparent preferences for Lys versus Cys between the two chemical classes, the potential for preferential reactivity was compared at the individual chemical level. In order to quantify differences in relative peptide reactivity, based on amino acid specificity, the ratio of Lys to Cys depletion (Lys:Cys ratio) was calculated for each test material. The Lys:Cys ratios for the respiratory and skin sensitizers are presented graphically in Figure 5. Values greater than one are indicative of increased relative reactivity to Lys, and values less than one demonstrate increased relative reactivity to Cys. Seventeen of the 20 respiratory allergens exhibited the ability to deplete both peptides, thus allowing for the calculation of the depletion ratio. The Lys:Cys ratio for the respiratory allergens ranged from a high of 36.6 for tetrachlorophthalic anhydride to a low of 0.22 for basic blue 99. Of the 48 skin sensitizers

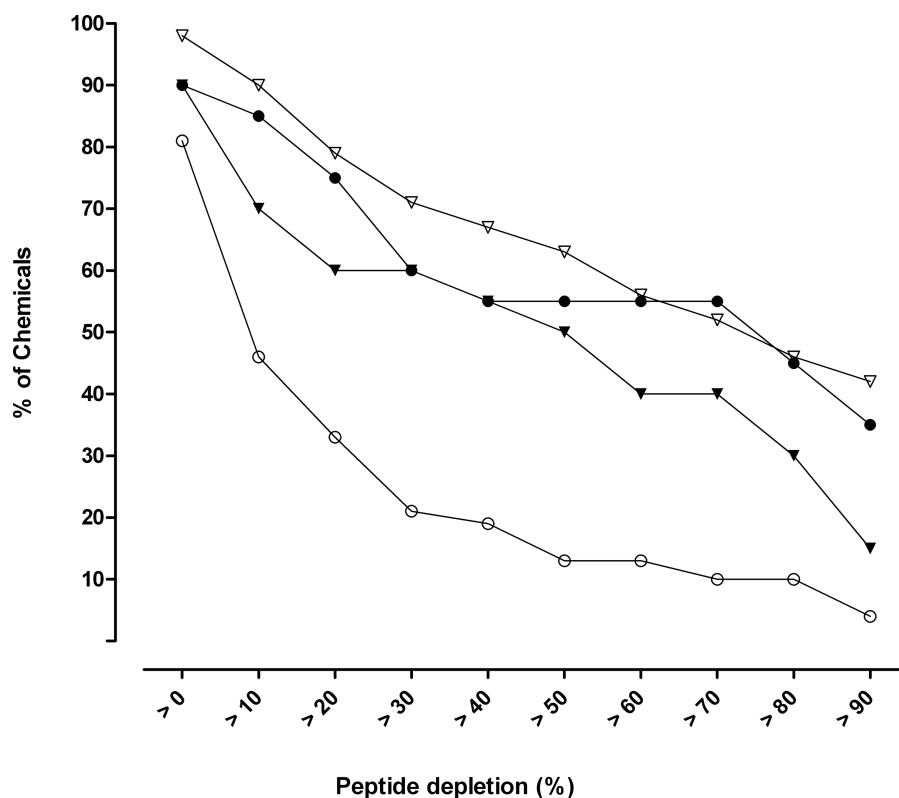


FIG. 4. Cumulative percentage distributions of peptide depletion data for a series of chemical respiratory and skin sensitizers reacted with either a Lys- or Cys-containing peptide. The cumulative percentage distributions were prepared on a “greater than” basis, where each interval represents the percent of test chemicals that resulted in depletion values greater than the lower bound of that interval. Statistically significant differences were observed across the distributions for the following comparisons: Cys versus Lys depletion within the skin allergens ($p < 0.05$); Lys depletion between the skin and respiratory allergens ($p < 0.05$). Lys depletion, respiratory (●) and skin (○) sensitizers; Cys depletion, respiratory (▼) and skin (▽) sensitizers.

examined, 39 were observed to deplete both peptides to some extent. The Lys:Cys ratio for these 39 materials was also calculated, the range for these materials spanned from a high of 15.0 for benzyl benzoate to a low of 0.016 for benzylideneacetone.

Based on the results presented in Figure 5, it is clear that the majority of skin-sensitizing chemicals exhibited higher relative Cys depletion when compared with the Lys peptide with a mean Lys:Cys ratio for the group of 0.9. The respiratory allergens tended toward higher relative Lys depletion with a mean Lys:Cys ratio of 4.6. A small number of respiratory sensitizers resulted in a Lys:Cys ratio of less than 1, this group comprised four diisocyanates (diphenylmethane diisocyanate, hexamethylene diisocyanate, toluene 2,4-diisocyanate, and toluene 2,6-diisocyanate) and basic blue 99. These trends are emphasized further, when it is appreciated, that the inability to calculate the Lys:Cys ratio for six skin sensitizers was due to a complete lack of reactivity to Lys, whereas each material maintained high levels of Cys depletion (thus the ratio could be considered as approaching zero). This included diphenylcyclopropanone, 2-mercaptobenzothiazole, 2-methyl-2H-isothiazol-3-one, and vinyl pyridine each of which exclusively depleted the Cys peptide at $> 90\%$; and

α -allylanisole and nonanoyl chloride depleting Cys at 68.3 and 18.2%, respectively. Additionally, α -amylcinnamaldehyde and benzyl benzoate were the only skin sensitizers with a Lys:Cys ratio significantly greater than 1. However, these two values may be considered “artificially” high, as the ratios were calculated based on very low levels of depletion for both the Lys and Cys peptides. The reported depletion for α -amylcinnamaldehyde (depletion of Lys = 3.9% and Cys = 0.6%) and benzyl benzoate (depletion of Lys = 3.0% and Cys = 0.2%) is sufficiently low that each may be considered a “false negative” in the DPRA based on practical experience with nonsensitizers. For all other materials (both skin and respiratory allergens), the Lys:Cys ratio was calculated with at least one peptide being depleted at greater than 10%.

DISCUSSION

It is clear from experimental studies and clinical experience that skin and respiratory chemical allergens are biologically distinct. For example, phthalic anhydride is a well-known cause of occupational asthma but, despite opportunities for occupational skin exposure, is not a significant cause of ACD

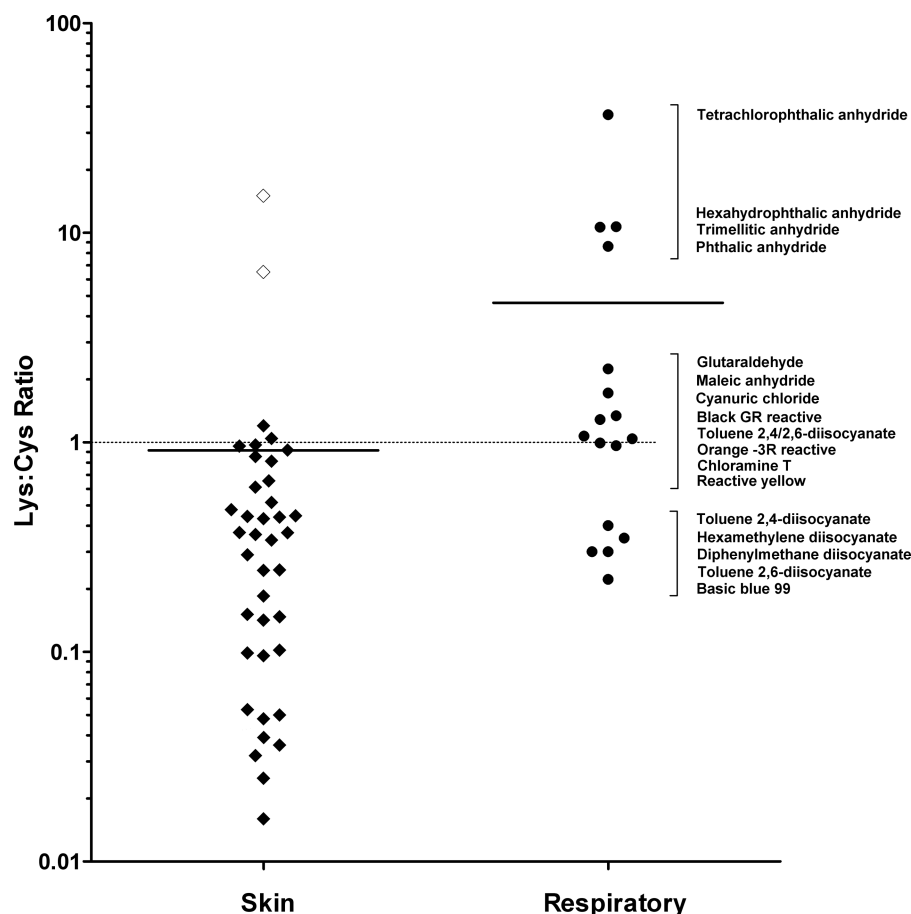


FIG. 5. Ratio of Lys to Cys peptide depletion data (Lys:Cys ratio) for a series of chemical respiratory and skin sensitizers in the DPRA. The ratios were calculated based on the average peptide depletion for the individual respiratory (●, $n = 17$) and skin sensitizers (◆, ◇; $n = 39$). The mean (—) Lys:Cys ratio for the skin sensitizers was 0.9 and for the respiratory allergens was 4.6. Although included in the comparisons, two skin sensitizers were considered to be potential outliers (◇). Their reactivity was sufficiently low to be considered a “false negative” in the DPRA: α -amylcinnamaldehyde (depletion of Lys = 3.9% and Cys = 0.6%) and benzyl benzoate (depletion of Lys = 3.0% and Cys = 0.2%). For all other materials, the Lys:Cys ratio was calculated with at least one peptide being depleted at greater than 10%.

in humans (Venables, 1989). DNCB, a well-established human skin allergen, has been demonstrated to elicit skin reactions following the induction of skin sensitization in animal models, whereas this material failed to provoke respiratory responses on inhalation challenge (Botham *et al.*, 1989). One interesting observation has been that respiratory allergens typically provoke positive responses in assays designed to identify skin sensitizers (such as the LLNA and guinea pig test methods), whereas these materials fail to cause ACD in humans (Dearman *et al.*, 2012). However, given that the endpoint in the LLNA is lymphocyte proliferation, a measure of immunogenicity that is independent of the quality of immune response provoked, it is perhaps not surprising that respiratory and skin sensitizers both provoke positive responses. From the perspective of hazard assessment the practical approach, based on the LLNA, has been to consider that positive results in the assay are best regarded as having 100% sensitivity and 0% specificity with respect to the identification of respiratory sensitizers. This has

highlighted further the pressing need for methods that will not only provide a means for the accurate identification of chemical respiratory allergens but also discriminate these from chemicals that have the potential to cause skin sensitization.

The available evidence indicates that skin and respiratory chemical allergens are associated with the elicitation of qualitatively discrete immune responses. Although there persists debate regarding the role of, and requirement for, IgE antibody, it does appear that allergic sensitization of the respiratory tract by chemicals is associated with the development of selective Th2-type immune responses (Kimber and Dearman, 2005). The reason for the development of selective immune responses to chemical allergens is not fully understood; however, the ability to covalently modify proteins and peptides is generally considered a prerequisite for the development of both forms of sensitization (Smith and Hotchkiss, 2001). It is possible that the characteristics of the hapten-protein conjugate may influence the quality of the immune response initiated, and therefore

one objective of these studies was to consider whether skin and respiratory chemical allergens display differences with regard to amino acid selectivity.

The results reported here demonstrate that the majority of respiratory allergens examined deplete the Lys- and Cys-containing peptides under the conditions of the DPRA. However, three of the allergens were observed either to lack reactivity altogether (chlorhexidine) or to have minimal reactivity (piperazine and ethylenediamine). It has been estimated that nearly a third of known skin sensitizers are nonelectrophilic requiring either metabolic or abiotic activation to form protein reactive intermediates; such chemicals have been termed prohaptens and prehaptens, respectively (Lepoittevin, 2006; Smith and Hotchkiss, 2001). A limitation of the DPRA, and many *in chemico* methods for hazard identification of skin-sensitizing chemicals, is the inability to reflect metabolic activation or abiotic transformations (Troutman *et al.*, 2011). This limitation may explain the low level of reactivity observed here with piperazine and ethylenediamine, as both materials have been suggested to react with Lys via Schiff base formation following metabolism and oxidation (Enoch *et al.*, 2010). Oxidative degradation is the most commonly cited abiotic transformation associated with sensitization; however, other pathways to activation are known to have a potential role. For example, Layton *et al.* (1987) demonstrated that chlorhexidine, although not directly reactive, could modify proteins following N-chlorination by environmental chlorine present in aqueous solutions at low levels (2 ppm). The requirement for such activation of chlorhexidine could account for the lack of reactivity observed here, given that the transformation would be unlikely under the experimental conditions of the DPRA. Despite the lack of reactivity by the three respiratory allergens above, all other test materials displayed levels of peptide depletion that provided for comparisons with the activity of skin-sensitizing chemicals.

Although other nucleophilic amino acids are available within proteins, the ϵ -amino group on Lys and the thiol of Cys are useful surrogates to investigate the chemical reactivity of haptens in the DPRA. Taken together, their utility is derived from an ability to act readily as nucleophiles to the major electrophilic chemical classes associated with sensitization, including Michael acceptors, S_NAr electrophiles, S_N2 electrophiles, Schiff base formers, and acylating agents (Aptula *et al.*, 2005). At first glance, these interactions would appear to be a purely chemical phenomenon. However, a number of researchers have suggested that preferential reactivity to specific amino acids may be associated with differing biological responses. For example, Meschkat *et al.* (2001) showed that the difference in skin-sensitizing potency between the strong sensitizer, alkenylsulfone, and the weak sensitizer, alkylsulfone, is best explained by the preference of the former for binding Lys in a model peptide containing a range of nucleophiles and within human serum albumin (HSA). In an intriguing series of experiments, Hopkins *et al.* (2005) demonstrated that chemical sensitizers

associated with Th1 cytokine secretion (indicative of skin allergens) selectively bound to the cellular proteins of U937 cells, whereas selective binding to serum proteins in the same experimental system was associated with respiratory allergens.

The data reported here demonstrate that chemical respiratory allergens, in general, preferentially react with the Lys peptide when compared with the peptide depletion measurements reported for the skin-sensitizing chemicals. Further, the data revealed that these same preferences were observed at the level of individual chemicals by examination of the Lys:Cys ratio. The Lys:Cys ratio is a potentially useful parameter because it provides a quantitative expression of the relative reactivity between the two peptides. But perhaps more importantly, it avoids difficulties that may arise from comparing differences in reactivity due to the presence of multiple functional groups on individual molecules resulting in the ability to react with or cross-link more than one peptide. The majority of respiratory allergens evaluated here included multiple reactive centers. This bias was self-selecting, as the presence of more than one functional group and the ability to cross-link proteins is known to be one factor that correlates with the ability of a chemical to induce respiratory allergy (Enoch *et al.*, 2010).

Although there was a clear trend for respiratory allergens to preferentially bind to Lys, the relationship was not always straight forward. This was most evident when comparing the anhydrides and diisocyanates, historically two of the most well described classes of respiratory sensitizers. Based on the Lys:Cys ratio, the anhydrides exhibited the greatest relative reactivity to Lys of all the respiratory allergens. These results are consistent with *in vivo* and *in vitro* studies that demonstrate anhydrides preferentially bind to Lys, whereas forming chemically unstable adducts with Cys (Jonsson *et al.*, 1995). Maleic anhydride showed a similar preference for Lys, although there was increased Cys reactivity when compared with other anhydrides. This increase may be due to maleic anhydride binding stably to Cys through a Michael addition on its readily reactive double bond, which is not accessible or present on the other anhydrides (Escalera and Palacian, 1989). Overall, the individual diisocyanates had low levels of Lys depletion relative to Cys resulting in a reactivity profile similar to the majority of skin sensitizers. The reaction chemistry associated with the diisocyanates is complex, and Lys may be a more significant nucleophile for this class of materials than would first appear. Fleischel *et al.* (2009) demonstrated that isocyanates can undergo a reversible conjugation to Cys thiols, and under competitive conditions transfer the isocyanate to amines such as Lys. This so-called "isocyanate exchange" can take place under physiologically relevant conditions *in vitro*; thus TDI-conjugated glutathione has been demonstrated to transfer selectively TDI to HSA Lys residues producing antigenic changes (Wisniewski *et al.*, 2011). The DPRA is limited to evaluating reactivity between a single test chemical and an individual nucleophile. The observation that the presence of multiple nucleophiles can have an impact on selective amino acid modifications is an area that deserves further research.

In addition to the diisocyanates, the reactive dyes and chloramine-T were observed to have Lys:Cys ratios within a similar range to that of some skin sensitizers. These respiratory allergens are not considered to be significant causes of ACD in humans; however, there are clinical case reports indicating that ACD can occur under comparatively rare circumstances (Rustemeyer *et al.*, 2012). To our knowledge, a mechanism has not been established linking directly the development of selective immune responses to the balance of Lys and Cys modifications within proteins. Although, the ability to react with Cys has been linked to the development of skin sensitization, specifically through the induction of the Nrf2-Keap1-ARE (nuclear factor erythroid 2-related factor 2, Kelch-like ECH-associated protein 1, antioxidant response element) regulatory pathway, a pathway regulated in part by specific binding to Cys on Keap1 (Natsch, 2010). It is proposed that effective development of qualitatively discreet immune responses may emerge through a combination of different exposure scenarios, specific modification of proteins and the activation of nucleophile dependent regulatory pathways (such as Nrf2-Keap1-ARE).

The work presented here has demonstrated that reactivity measurements, such as those obtained in the DPRA, could provide useful information to assist in the identification of potential respiratory allergens. From a risk assessment/management perspective, it is desirable to separate chemicals based on their potential to act as skin or respiratory sensitizers. To this end, we have introduced the Lys:Cys ratio as one example of a quantitative descriptor that has the potential to assist in differentiation. It is important to emphasize that the DPRA, and readouts such as the Lys:Cys ratio, cannot be expected alone to predict and characterize potential respiratory allergens. Rather, the value of such readouts lies in their potential to be incorporated into tiered assessment and testing strategies. This work has focused on the most commonly cited materials associated with occupational asthma and rhinitis; however, other important classes (such as the penicillins) and individual materials (such as plicatic acid) have been described (Chan-Yeung and Malo, 1994; HSE, 2001). The dataset reported here provides a useful benchmark to evaluate the selectivity of additional chemical sensitizers in the DPRA, and remains a focus of future work. Ultimately, it is anticipated that increased understanding of the reactivity profile of both respiratory and skin sensitizers will inform the development of predictive tools for the identification and characterization of respiratory allergens.

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