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Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach

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In the interest of reducing animal use, *in vitro* alternatives for skin sensitization testing are under development. One unifying characteristic of chemical allergens is the requirement that they react with proteins for the effective induction of skin sensitization. The majority of chemical allergens are electrophilic and react with nucleophilic amino acids. To determine whether and to what extent reactivity correlates with skin sensitization potential, 82 chemicals comprising allergens of different potencies and non-allergenic chemicals were evaluated for their ability to react with reduced glutathione (GSH) or with two synthetic peptides containing either a single cysteine or lysine. Following a 15-min reaction time with GSH, or a 24-h reaction time with the two synthetic peptides, the samples were analyzed by high-performance liquid chromatography. UV detection was used to monitor the depletion of GSH or the peptides. The peptide reactivity data were compared with existing local lymph node assay data using recursive partitioning methodology to build a classification tree that allowed a ranking of reactivity as minimal, low, moderate, and high. Generally, nonallergens and weak allergens demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent allergens displayed moderate to high peptide reactivity. Classifying minimal reactivity as nonsensitizers and low, moderate, and high reactivity as sensitizers, it was determined that a model based on cysteine and lysine gave a prediction accuracy of 89%. The results of these investigations reveal that measurement of peptide reactivity has considerable potential utility as a screening approach for skin sensitization testing, and thereby for reducing reliance on animal-based test methods.

Key Words: **allergens; alternatives; skin sensitization; peptide reactivity; prediction model.**

Allergic contact dermatitis (ACD) resulting from skin sensitization is a critical toxicological endpoint evaluated for all new chemicals developed for consumer and/or occupational use. The acquisition of skin sensitization and the subsequent elicitation of an ACD reaction in the skin are processes dependent upon recognition of chemical allergens in the skin by Langerhans cells (LC) and the induction of specific T lymphocyte responses (Kimber *et al.*, 2000). The local lymph node assay (LLNA) is viewed as the most appropriate skin sensitization test method for the evaluation of chemicals that have potential to come in contact with the skin (Cockshott *et al.*, 2006). The LLNA is based upon characterization of induced proliferative responses in draining lymph nodes following topical exposure of mice to chemicals (Gerberick *et al.*, 2000; Kimber *et al.*, 2002). However, there is a critical need to develop non-animal–based methods for the evaluation of new chemicals that will reduce significantly or eliminate the need for animals in skin sensitization testing in the future (Jowsey *et al.*, 2006; Ryan *et al.*, 2001, 2005). This is of particular importance in view of the forthcoming European Union ban on *in vivo* testing of cosmetic and toiletry ingredients following the publication of the Seventh Amendment to the Cosmetic Directive (European Union Seventh Amendment to Cosmetic Directive) and for Registration, Evaluation, and Authorization of Chemicals that requires evaluation of a large number of chemicals.

Fortunately, the underlying chemical and cellular mechanisms of ACD are relatively well understood to aid scientists in the development of alternative methods for skin sensitization testing. It is believed that for a chemical to function as a contact sensitizer (or allergen), it must be capable of penetrating into the viable epidermis, react with protein, induce local trauma, and be recognized by the immune system. Thus, characterization of skin sensitization must integrate various sources of information from a battery of assays representing the key steps of skin allergy (Jowsey *et al.*, 2006). For example, investigators have undertaken recently the development of chemical reactivity screening methods for aiding in the assessment of a chemical's skin sensitization potential (Aptula *et al.*, 2006; Divkovic *et al.*, 2005; Gerberick *et al.*, 2004; Kato *et al.*, 2003; Natsch *et al.*, in press).

The correlation of skin protein reactivity and skin sensitization is well established and has been known for many years (Dupuis and Benezra, 1982; Landsteiner and Jacobs, 1936;

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Lepoittevin *et al.*, 1998). It is accepted that if a chemical is capable of reacting with protein directly or after appropriate biotransformation, then it has the potential to act as an allergen. While a variety of mechanisms contribute to protein reactivity, it is generally recognized that this process involves the reaction of a small molecule, having electrophilic properties, with a nucleophilic amino acid on a protein. The majority of chemical allergens (or their metabolites) have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups, nucleophiles, capable of reacting with electrophilic allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can also react with electrophiles (Ahlfors *et al.*, 2003; Dupuis and Benezra, 1982; Lepoittevin *et al.*, 1998).

Measuring chemical reactivity on nucleophile-containing peptides has potential utility for evaluating the skin sensitization potential of chemicals (Gerberick *et al.*, 2004). Specifically, it was demonstrated that peptides containing either cysteine or lysine along with glutathione (GSH) served as surrogate nucleophiles to quantitatively measure chemical reactivity. The purpose of this work was to examine the reactivity of a large set of test chemicals (38 from original study and 44 new chemicals for a total of 82) using lysine, cysteine, and GSH peptides at different peptide to chemical molar ratios to determine whether the degree of reactivity correlated with the compound's sensitization potency. The data were analyzed using a classification tree model approach to develop a pragmatic prediction model for assessing and interpreting the peptide reactivity assay data.

MATERIALS AND METHODS

Test chemicals. The rationale for selecting chemicals for evaluation was based on chemical diversity and on the availability of robust LLNA data representing a good distribution of weak, moderate, strong, and extreme allergens along with nonallergens. The following chemicals with accompanying purity and CAS numbers were purchased from Aldrich Chemical Company (Milwaukee, WI): 2-acetylcyclohexanone, 97% (874-23-7); a-amylcinnamaldehyde, 85% (122-40-7); benzaldehyde, 95% (100-52-7); 1,2-benzisothiazolin-3-one, 97% (2634-33-5); benzyl benzoate, 99% (120-51-4); benzylideneacetone, 99% (122-57-6); 1-butanol, 99.5% (71-36-3); chlorobenzene, 99% (108-90-7); cinnamaldehyde, 99% (14371-10-9); coumarin (91-64-5); cyclamen aldehyde, 90% (103-95-7); diethyl maleate, 97% (141-05-9); diethyl phthalate, 99.5% (84-66-2); diphenylcyclopropenone, 98% (886-38-4); ethyl acrylate, 99% (140-88-5); ethyl vanillin, 99% (121-32-4); ethyleneglycol dimethacrylate, 98% (97-90-5); farnesal, 85% (19317-11-4); formaldehyde (50-00-0); 2,4-heptadienal, 90% (5910-85-0); hexenal, 98% (6728-26-3); a-hexylcinnamaldehyde, 85% (101-86-0); 4-hydroxybenzoic acid, 99% (99-96-7); hydroxycitronellal, 95% (107-75-5); 2-hydroxyethyl acrylate, 96% (818-61-1); 2-hydroxypropyl methacrylate, 97% (923-26-2); 2-mercaptobenzothiazole, 98% (149-30-4); 6-methyl coumarin, 99% (92-48-8); methyl salicylate, 99% (119-36-8); methyl-2-nonynoate, 99% (111-80-8); methylparaben, 99% (99-76-3); metol, 99% (55-55-0); nonanoyl chloride, 96% (764-85-2); oxalic acid, 99% (144-62-7); oxazolone, 90% (15646-46-5); perillaldehyde, 92% (2111-75-3); phenylacetaldehyde, 90% (122-78-1); 2-phenylpropionaldehyde, 98% (93-53-8); phthalic anhydride, 99% (85-44-9); propyl gallate, 98% (121-79-9); propyl paraben, 99% (94-13-3); resorcinol, 99.5% (108-46-3); salicylic acid, 99% (69-72-7); squaric acid, 99% (2892-51-5); vanillin, 99% (121-33-5); vinylidene dichloride, 99% (75-35-4); vinyl pyridine (1337-81-1).

The following chemicals with accompanying purity and CAS numbers were purchased from Sigma Chemical Company (St Louis, MO): *p*-benzoquinone, 98% (106-51-4); CD3 (25646-71-3); 2,4-dinitrochlorobenzene, 99% (97-00-7); glutaraldehyde, 70% (111-30-8); imidazolidinyl urea, 95% (39236-46-9); isopropanol, 99% (67-63-0); isopropyl myristate, 98% (110-27-0); lactic acid, 85% (50-21-5); 1-(4-methoxyphenyl)-1-penten-3-one (104-27-8); nonanoic acid, 97% (112-05-0); octanoic acid, 98% (124-07-2); sulfanilamide, 99% (63-74-1); sulphanilic acid, 99% (121-57-3); trimellitic anhydride, 97% (552-30-7).

The following chemicals with accompanying purity and CAS numbers were purchased from Fluka Chemical Company (Milwaukee, WI): 4-allylanisole, 98% (140-67-0); benzoyl peroxide, 97% (94-36-0); 1-bromobutane, 99% (109-65-9); 2,3-butanedione, 99% (431-03-8); 5-chloro-2-methyl-4-isothiazolin-3-one (26172-55-4); ethylbenzoylacetate, 97% (94-02-0); fluorescein isothiocynate, 98% (3326-32-7); glyoxal (107-22-2); lilial, 95% (80-54-6); 4-methoxyacetophenone, 99% (100-06-1); palmitoyl chloride, 98% (112-67-4); propylene glycol, 99.7% (57-55-6); 2,2,6,6-tetramethyl-3,5-heptanedione, 98% (1118-71-4).

Hexane (110-54-3) was purchased from EM Science (Gibbstown, NJ). Bandrowski's base was purchased from ICN (Costa Mesa, CA). Glycerol, 99% (56-81-5) was purchased from J.T Baker (Phillipsburg, NJ).Tetracholorosalicylanilide (1154-59-2) was purchased from Eastman Kodak Company (Rochester, NY). Lauryl gallate, 98% (1166-52-5) was purchased from Alfa Aesar (Ward Hill, MA). 5-Methyl-2,3-hexandione (13706-86-0) was purchased from Penta MFG (Livingston, NJ). Kathon CG (55965-84-9) was purchased from Rohm & Haas (Philadelphia, PA). 2-Methyl-2H-isothiazol-3-one (2682-20-4) was supplied by J.-P. L.

LLNA protocol and chemicals tested. The LLNA data reported in this manuscript are derived from previously conducted studies. The LLNA studies were conducted as described elsewhere (Gerberick *et al.*, 2000; Kimber *et al.*, 2002). Briefly, groups of CBA female mice (7–12 weeks of age) were exposed topically on the dorsum of both ears to 25 μl of test material or to an equal volume of the relevant vehicle alone. Treatment was performed daily for three consecutive days. Five days following the initiation of exposure, all mice were injected via the tail vein with 250 μl of phosphate-buffered saline containing 20 μCi of tritiated thymidine. Mice were sacrificed 5 h later, and the draining lymph nodes were excised for each experimental group. The incorporation of tritiated thymidine measured by β-scintillation counting was reported in disintegrations per minute (dpm). A stimulation index (SI) was calculated for each allergen-treated group as the ratio of the dpm of the treated group over the dpm of the concurrent vehicle control. A substance was classified as a skin sensitizer if at one or more test concentrations it induced a threefold or greater increase in local lymph node proliferative activity compared with concurrent vehicle-treated controls.

Potency estimation in the LLNA. The method used to determine the relative skin sensitization potency of a chemical has been previously described and is based upon the mathematical estimation of the concentration necessary to induce a threefold increase in the proliferative activity in the draining lymph nodes relative to vehicle-treated mice (Basketter *et al.*, 1999). This estimated concentration, known as the EC3 value, is calculated by conducting a linear interpolation of coordinates above and below the value of three on the LLNA dose-response plot. The EC3 value for chemicals which had an SI greater than three for the lowest concentration tested was extrapolated from the two lowest doses evaluated (Ryan *et al.*, in press). EC3 values extrapolated by this method were calculated by log-linear interpolation between these two points on a plane in which the dose level and SI are represented on the x-axis and y-axis, respectively. Existing dose-response data from previously conducted LLNA experiments have been used to calculate the EC3 values for the chemicals used

in this manuscript. An arbitrary classification scheme based on EC3 values was used for categorizing the relative skin sensitization potency of chemicals evaluated in this study (Kimber *et al.*, 2003). This system classifies the sensitization potency of a chemical as extreme (EC3 < 0.1), strong (EC3 ≥ 0.1 to < 1), moderate (EC3 ≥ 1 to < 10), weak (EC3 ≥ 10 to ≤ 100), and nonsensitizing are not calculated.

The specific EC3 potency data used in this paper for the majority of chemicals are found in a recently published LLNA database paper (Gerberick *et al.*, 2005). References for the other chemicals used are as follows: diphenylcyclopropenone (Ryan *et al.*, 2000), phthalic anhydride (Dearman *et al.*, 1992), oxazolone (Loveless *et al.*, 1996), propyl gallate (Ashby *et al.*, 1995; Basketter and Scholes, 1992), metol (Ashby *et al.*, 1995; Basketter and Scholes, 1992), benzoyl peroxide (Kimber *et al.*, 1998), squaric acid (Ryan *et al.*, 2000), 2-methyl-2H-isothiazol-3-one (Estrada *et al.*, 2003), lilial (Basketter *et al.*, 2001), and nonanoic acid (P&G, unpublished data).

GSH, cysteine, and lysine peptide depletion assays. A method to measure reactivity of a test chemical with reduced GSH was recently developed (Gerberick *et al.*, 2004) which is based on a previously described method (Farriss and Reed, 1987). Briefly, 50 µl of a 2mM GSH stock solution prepared in oxygen-free 100mM sodium phosphate buffer (pH 7.4) and 50 µl of a 200mM test chemical prepared in dimethyl sulfoxide (DMSO) were added to 400 µl of oxygen-free 100mM sodium phosphate buffer (pH 7.4). The final reaction, containing 0.2mM GSH and 20mM of the test chemical, representing 1:100 molar ratio, was mixed and incubated for 15 min at 25°C with agitation. Control samples and standards used for defining the calibration curve for each analysis were prepared without test chemical for GSH (0.05–200mM) and glutathione disulfide (GSSG) (0.025–100mM). All samples were prepared in triplicate. Following incubation, GSH and GSSG in the samples and standards were derivatized with iodoacetic acid and 2,4 dinitrofluorobenzene. Derivatized GSH and GSSG were separated and quantitated by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Alliance 2695 system (Waters Corporation, Milford, MA) using a Waters UV detector (365 nm) and a Waters Spherisorb NH_2 analytical column (3 µm, 2.0 × 100 mm) under gradient conditions. Total GSH (GSH equivalents as GSH or GSSG) in each sample was determined from the calibration curve and used to calculate the percent peptide depletion relative to the mean concentration of total GSH in the control sample (no test chemical).

A method to measure reactivity of a test chemical with model heptapeptides containing lysine (Ac-RFAAKAA-COOH) or cysteine (Ac-RFAACAA-COOH) was recently developed (Gerberick *et al.*, 2004). Peptides were prepared and purified by the SynPep Corporation (Dublin CA, USA) to > 90% purity as measured by HPLC, and molecular weight confirmation was determined by flow injection positive-ion electrospray mass spectrometry. Briefly, 400 µl of a 1.25mM peptide stock solution prepared in buffer and a 100mM test chemical stock solution prepared in either acetonitrile or DMSO/acetonitrile were added to 100mM ammonium acetate buffer (pH 10.2) for the lysine peptide or 100mM sodium phosphate buffer (pH 7.5) for the cysteine peptide. The final reaction, containing 0.5mM of the peptide and 5 or 25mM of the test chemical, representing 1:10 and 1:50 molar ratios, was mixed and incubated in the dark for 24 h at 25°C. Control samples and standards used for defining the calibration curve for each analysis were prepared without test chemical for each peptide and ranged from 0.0156 to 1.0mM. All samples were prepared in triplicate. Following incubation, the peptide was quantified by reverse-phase HPLC (Waters 2695 Alliance) on a Zorbax SB-C18 column (3.5 µm, 100 × 2.1 mm) with UV detection at 220 nm (Waters 996 PDA detector) using an external standard linear calibration curve. The UV spectrum was collected from 210 to 400 nm to permit verification of the peptide peak identity. Peptide reactivity was reported as percent depletion based on the decrease in nonreacted peptide concentration in the sample relative to the average concentration measured in the control.

Classification tree model development. The goal was to develop a prediction model that would quantify in some way peptide depletion as related to level of reactivity. Various models were developed using classification tree methodology (Brieman *et al.*, 1983) and the recursive partitioning routines implemented in S-Plus 7.0 statistical software (2003, Insightful Corp., Seattle, WA). During model development, each peptide at each concentration (GSH 1:100, cysteine 1:10, cysteine 1:50, lysine 1:10, and lysine 1:50) was considered as a potential predictor. For each of the 82 chemicals examined, three measurements were taken as the percentage of peptide depletion for each peptide/concentration, and the average depletion was determined. Some potential models were developed using these peptide depletion averages as predictors, while other potential models were developed using peptide depletion percentages averaged further across various peptides/concentrations (e.g., the average of the cysteine 1:10 and lysine 1:50 percentages). Specifically, models were developed using the following peptide depletion values as potential predictors in each model: model #1, average of all five peptides as the only predictor; model #2, cysteine 1:10, cysteine 1:50, GSH 1:100, lysine 1:10, lysine 1:50; model #3, average of cysteine 1:10, GSH 1:100, and lysine 1:10 as the only predictor; model #4, average of cysteine 1:10, cysteine 1:50, lysine 1:10, and lysine 1:50 as the only predictor; model #5, average of cysteine 1:10 and lysine 1:50 as the only predictor; model #6, cysteine 1:10, cysteine 1:50, lysine 1:10, lysine 1:50.

Classification tree building begins with the root node, which includes all of the chemical compounds in the learning data set (a total of 56). Beginning with this node, if more than one variable is considered, S-Plus software finds the best possible variable (peptide and concentration) to split the node into two child nodes. In order to find the best peptide/concentration, the software checks all possible peptides/concentrations as well as all possible values of the peptide/concentration used to split the node. For example, suppose that an attempt is made to build a tree using cysteine 1:10 and lysine 1:10. For each of these peptides, the individual chemicals are rank ordered based on their depletion values as potential predictors. The root node is then split into two child nodes using the average of two adjacent values of one of the peptides. The rank-ordered cysteine 1:10 values are − 10, − 3.8, − 3.7, − 1.9, − 1.3, . . . , 100, so child nodes are created by splitting the root node based on cysteine 1:10 at − 6.9 (the average of − 10 and − 3.8), − 3.75 (the average of − 3.8 and − 3.7), etc. One child node represents all chemicals with peptide depletion less than the specified value, and the other node represents all chemicals with peptide depletion greater than the specified value. The software then seeks to maximize the average ''purity'' of the two child nodes. In other words, a pair of child nodes in which one node contains all nonsensitizers and weak sensitizers and the other node contains all moderate and strong sensitizers would be superior to a pair of child nodes in which each node contains a mix of chemicals from each sensitization category. Once the best pair of child nodes is determined, the process that was used on the root node is repeated on each child node. The splitting of nodes into child nodes continues in an iterative manner until the level of purity in the child nodes reaches a reasonable level or until a minimum sample size per node is reached.

In the various models fit to peptide reactivity data, splits were made until there were a total of four child nodes. Once these nodes were determined, each node was named based on the sensitization category most often represented in each node. The names assigned to each node include ''minimal reactivity,'' ''low reactivity,'' ''moderate reactivity,'' or ''high reactivity'' (corresponding to the prediction of non-, weak, moderate, and strong/extreme sensitizers, respectively). Chemicals of a different sensitization category than the category of the node in which they are included are considered to be misclassified.

This modeling procedure was conducted six times (once for each potential model previously mentioned) based on all 56 chemicals in the learning data set. Once the six models were determined, they were tested on 26 additional chemicals. Some advantages to using classification trees for prediction include ease of variable selection and model interpretation, no assumptions regarding the distribution of the data, predictor interaction effects are taken into account, models can discriminate on one or more variables, differing costs can be taken into account for different types of misclassification, and more than two response levels are easily handled.

The final classification tree model was assessed via scatterplots and by calculating Cooper statistics (Cooper *et al.*, 1979). Cooper statistics were used to determine how well the model distinguished sensitizers (of any strength) and

TABLE 1
Reactivity of Chemical Substances to GSH or Synthetic Peptides with Results Expressed as Percent Depletion of Nonreacted Peptide

Concentration of peptide:concentration of test substance	GSH 1:100 (0.2mM:20mM)		Lysine 1:10 (0.5mM:5mM)		Lysine 1:50 (0.5mM:25mM)		Cysteine 1:10 (0.5mM:5mM)		Cysteine 1;50 (0.5mM:25mM)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Strong/extreme										
Diphenylcyclopropenone	22.0	7.5	0.3	4.1	− 0.7	3.8	98.8	2.0	100.0	0.0
Oxazolone	22.6	9.5	42.9	3.2	49.6	1.8	75.5	1.4	89.3	2.6
Benzoyl peroxide	100.0	0.0	28.6	8.1	81.3	2.9	100.0	0.0	80.6	3.7
Kathon CG	46.7	9.3	4.5	1.0	3.9	1.0	99.1	1.6	99.5	0.9
Bandrowski's base	30.0	9.3	11.6	2.5	4.2	17.0	87.5	0.3	96.3	0.1
5-Chloro-2-methyl-4-isothiazolin-3-one	74.7	8.5	3.9	3.2	35.1	14.0	96.3	2.8	87.8	6.0
p-Benzoquinone	100.0	0.0	55.6	3.0	91.0	0.2	99.0	1.8	97.1	2.8
Tetrachlorosalicylanilide	0.7	2.3	− 0.2	0.7	9.0	24.0	36.8	20.0	96.5	0.7
2,4 Dinitrochlorobenzene	43.6	2.6	13.4	9.0	14.7	4.2	100.0	0.0	100.0	0.0
Glutaraldehyde	20.8	4.0	66.0	2.2	85.4	3.5	30.2	0.5	70.0	4.7
Fluorescein isothiocynate	92.6	1.5	15.5	0.3	61.1	1.5	100.0	0.0	100.0	0.0
Phthalic anhydride	100.0	0.0	9.9	0.8	75.0	3.9	− 1.9	1.0	− 5.5	2.0
Lauryl gallate	42.2	13.6	6.8	0.6	8.7	4.2	90.9	13.1	100.0	0.0
Propyl gallate	19.7	4.3	13.5	11.7	26.6	10.7	59.9	35.2	97.7	2.4
CD3	63.6	13.6	18.9	2.5	13.6	0.5	90.1	1.1	83.0	1.1
Trimellitic anhydride	97.6	4.0	6.5	0.7	43.7	4.9	− 1.1	5.7	− 14.8	5.7
Formaldehyde	37.5	3.5	0.7	0.6	11.2	3.5	60.4	4.1	75.0	3.0
Metol	86.1	3.4	34.2	3.8	44.7	3.8	100.0	0.0	38.3	3.1
Moderate										
2-Hydroxyethyl acrylate	98.1	1.8	38.2	2.4	88.9	0.3	92.6	0.5	92.2	0.1
Glyoxal	33.0	6.3	29.7	6.2	67.8	1.9	56.5	1.7	94.0	8.5
Vinyl pyridine	38.0	0.7	0.1	11.3	− 16.9	16.2	92.1	0.4	90.3	0.1
2-Mercaptobenzothiazole	24.0	5.9	− 1.9	1.2	− 3.0	0.6	97.5	4.2	99.2	0.7
Nonanoyl chloride	79.0	13.0	− 1.1	9.3	− 6.3	1.8	18.2	3.0	23.0	11.0
2-Methyl-2H-isothiazol-3-one	73.0	5.8	2.6	9.4	− 5.6	5.2	97.9	0.3	100.0	0.0
1,2-Benzisothiazolin-3-one	14.5	1.3	—	—	9.7	2.5	97.7	0.1	83.5	1.6
Methyl-2-nonynoate	92.7	4.1	2.5	2.9	3.2	4.0	100.0	0.0	100.0	0.0
Cinnamaldehyde	46.7	5.2	27.5	1.7	43.2	4.1	70.6	1.0	88.6	1.4
Phenylacetaldehyde	− 4.7	0.7	12.9	0.5	22.6	1.9	60.7	13.3	81.1	3.7
Benzylideneacetone	58.5	3.9	− 2.2	0.5	1.5	0.9	94.7	2.3	96.5	3.0
2,4-Heptadienal	93.0	2.5	19.8	3.5	23.9	5.0	97.3	0.1	93.4	2.7
Squaric acid	16.5	4.0	3.2	1.3	4.8	4.9	46.9	8.7	94.3	4.2
Trans-2-hexenal	68.0	3.9	2.8	1.8	3.6	2.6	97.9	0.3	93.0	1.0
Diethyl maleate	83.3	4.5	33.4	0.6	85.5	1.6	100.0	0.0	100.0	0.0
2-Phenylpropionaldehyde	3.7	3.9	8.8	2.1	21.2	1.6	48.2	7.1	100.0	0.0
Perillaldehyde	10.2	4.7	13.3	0.5	13.8	0.5	31.9	3.3	85.0	0.7
Palmitoyl chloride	77.0	14.1	0.2	0.4	26.6	1.3	25.5	6.6	60.1	5.2
1-(4-Methoxyphenyl)-1-penten-3-one	− 0.2	1.5	8.3	2.3	14.3	3.2	29.9	5.6	75.8	12.6
Weak										
α-Hexylcinnamaldehyde	− 2.6	3.2	1.0	1.5	− 1.6	2.9	− 0.3	1.2	1.0	2.4
α-Amyl cinnamaldehyde	0.2	10.1	2.2	1.2	3.9	1.5	0.6	0.2	0.7	10.6
2,3-Butanedione	0.5	4.1	23.7	1.3	27.0	3.9	79.0	20.8	75.5	16.8
Farnesal	10.0	2.6	5.9	0.6	8.5	13.6	16.4	3.5	71.1	6.7
Oxalic acid	− 2.9	3.1	0.0	1.4	− 0.9	0.7	0.9	5.8	− 5.8	7.7
Benzyl benzoate	0.7	5.5	2.9	0.9	3.0	5.3	0.2	1.1	− 2.2	5.5
4-Allylanisole	17.8	3.1	− 0.9	1.3	− 0.8	1.8	20.6	5.6	61.5	5.4
Lilial	7.7	0.8	0.8	0.8	0.7	0.2	14.0	6.4	71.6	15.5
Cyclamen aldehyde	10.4	5.5	0.3	0.4	1.0	0.4	18.9	8.1	46.1	9.7
Imidazolidinyl urea	30.7	3.0	0.2	1.0	1.3	1.9	52.3	6.0	74.7	2.3
5-methyl-2,3-hexandione	− 2.6	9.9	5.0	1.1	7.5	1.1	25.8	4.0	69.6	7.3
2,2,6,6-Tetramethyl-3,5-heptanedione	5.4	8.2	0.6	1.9	0.0	0.2	1.4	13.6	− 3.7	0.6
Ethyleneglycol dimethacrylate	3.6	5.6	4.5	1.6	12.4	3.0	87.3	5.0	100.0	0.0

TABLE 1—*Continued*

Concentration of peptide:concentration of test substance	GSH 1:100 (0.2mM:20mM)		Lysine 1:10 (0.5mM:5mM)		Lysine 1:50 (0.5mM:25mM)		Cysteine 1:10 (0.5mM:5mM)		Cysteine 1;50 (0.5mM:25mM)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Ethyl acrylate	89.8	− 4.5	24.0	20.7	93.7	1.3	96.4	0.3	97.6	2.1
Hydroxycitronellal	− 1.8	3.9	10.6	1.2	6.5	2.0	17.5	1.7	55.8	3.6
Nonsensitizers										
Glycerol	1.2	4.2	− 0.6	1.2	2.1	0.9	− 3.8	5.2	0.9	1.9
Hexane	− 0.8	4.1	− 0.7	0.3	− 5.1	0.6	− 0.4	0.8	0.3	2.3
Diethyl phthalate	10.9	13.3	0.7	1.0	− 0.7	0.9	0.8	1.7	3.3	4.6
Octanoic acid	− 1.6	3.1	− 0.3	0.7	0.9	0.1	− 1.0	0.7	2.7	3.7
2-Hydroxypropyl methacrylate	5.5	4.8	—	—	− 13.6	7.8	58.4	5.9	96.5	1.5
1-Butanol	6.1	7.5	1.2	2.5	1.2	0.8	− 0.4	1.4	− 4.1	4.3
4-Hydroxybenzoic acid	− 1.0	5.8	2.2	1.2	2.2	2.1	− 0.3	0.8	14.0	14.0
6-Methyl coumarin	− 1.6	8.6	0.2	2.5	4.0	5.6	1.4	0.3	− 0.3	3.9
Methyl salicylate	4.2	3.5	2.4	0.8	1.6	0.3	0.3	0.8	0.8	7.7
Chlorobenzene	3.2	2.3	1.4	0.8	1.3	0.2	0.4	0.2	− 2.7	2.2
Lactic acid	− 1.1	11.1	3.2	0.4	0.8	0.5	− 0.9	0.3	11.5	21.0
1-Bromobutane	4.0	3.3	0.2	1.2	1.2	0.4	13.8	3.6	47.6	24.1
2-Acetylcyclohexanone	4.3	4.1	− 4.6	2.2	12.5	0.5	18.2	4.4	40.8	8.5
4-Methoxyacetophenone	2.5	3.2	− 0.8	0.7	0.1	0.3	4.7	5.0	− 3.3	1.4
Ethylbenzoylacetate	3.9	3.0	− 0.6	0.6	1.9	0.4	2.3	5.5	0.5	0.5
Ethyl vanillin	− 0.7	3.1	—	—	9.7	5.5	1.1	17.0	—	—
Isopropanol	1.4	6.8	− 1.3	0.1	0.5	0.5	− 10.0	17.0	− 3.1	0.3
Propylene glycol	4.2	2.5	0.2	2.0	0.6	0.7	− 0.9	17.5	− 3.0	0.6
Sulfanilamide	12.8	4.5	0.3	1.6	0.8	0.5	− 1.3	17.3	− 2.1	0.2
Isopropyl myristate	4.9	− 8.7	3.5	2.5	− 4.0	17.3	0.8	1.7	− 2.2	2.9
Benzaldehyde	6.8	2.6	− 1.5	1.2	− 1.7	1.4	7.2	8.8	− 2.2	2.6
Methylparaben	3.4	4.2	− 0.6	2.3	− 0.4	0.8	3.6	6.8	− 5.4	6.3
Nonanoic acid	4.0	6.5	− 4.1	3.9	− 9.6	2.9	− 3.7	6.1	5.2	4.6
Propyl paraben	− 1.0	6.0	− 0.7	0.2	− 0.2	1.3	8.2	2.3	21.8	6.3
Resorcinol	3.6	6.2	− 0.9	1.7	− 0.8	1.9	1.6	5.6	2.3	2.0
Salicylic acid	− 8.2	− 5.2	− 6.9	2.7	—	—	3.5	4.2	9.3	5.6
Sulphanilic acid	− 6.0	2.3	− 0.3	1.6	0.5	1.0	5.3	5.5	1.4	4.1
Vanillin	1.5	4.7	0.2	2.0	− 6.6	3.6	3.2	5.5	34.2	5.1
Coumarin	1.0	3.8	− 9.9	2.9	− 14.9	22.0	1.0	4.6	− 14.5	10.1
Vinylidene dichloride	.0	5.3	− 0.8	7.8	− 4.3	18.2	2.4	1.7	4.0	1.7

nonsensitizers. The Cooper statistics calculated include sensitivity (the proportion of true sensitizers predicted as having low, moderate, or high reactivity), specificity (the proportion of true nonsensitizers predicted as having minimal reactivity), positive predictivity (the proportion of chemicals classified as having low, moderate, or high reactivity that are true sensitizers), negative predictivity (the proportion of chemicals classified as having minimal reactivity that are true nonsensitizers), and accuracy (the overall proportion of correct predictions). Cooper statistics were computed on the entire set of chemicals (up to 56 training set chemicals and 26 validation set chemicals).

RESULTS

Peptide Reactivity Data with GSH, Lysine, and Cysteine

Peptide depletion results on 38 chemicals using GSH, cysteine, and lysine peptides were previously published (Gerberick *et al.*, 2004). The ratios of peptide to chemical used were 1:100 for GSH, 1:50 for lysine, and 1:10 for cysteine. The results indicated a strong correlation between allergen potency and depletion of the nonreacted peptide. In this study, we have expanded the number of chemicals evaluated to 82 and added two experimental conditions: cysteine at 1:50 and lysine at 1:10. The results for the 82 test chemicals are presented in Table 1. The chemicals are listed in the order of lowest EC3 values (i.e., the most potent allergens) through nonsensitizers and include 18 extreme/strong sensitizers; 19 moderate sensitizers; 15 weak sensitizers; and 30 nonsensitizers based on an existing LLNA categorization scheme (Kimber *et al.*, 2003). The LLNA EC3 data reported in this manuscript are derived from previously conducted studies (Ashby *et al.*, 1995; Basketter and Scholes, 1992;

Basketter *et al.*, 2001; Dearman *et al.*, 1992; Estrada *et al.*, 2003; Gerberick *et al.*, 2005; Kimber *et al.*, 1998; Loveless *et al.*, 1996; Ryan *et al.*, 2000). Generally, it is evident that the more potent the allergen, the more peptide depletion that is observed (Table 1), specifically for the GSH and cysteine peptides. For the majority of the extreme and strong allergens, greater than 75% depletion was observed for the 1:50 cysteine peptide. Less peptide depletion was noted for the GSH and 1:10 cysteine peptides but again generally more depletion was observed with the more potent allergens. With 1:10 and 1:50 lysine peptides, peptide depletion was greater also with the more potent allergens but not to levels of the cysteine-containing peptides. Interestingly, phthalic anhydride and trimellitic anhydride demonstrated significant depletion with GSH and lysine 1:50 peptides but not with the others. Finally, only a few of the nonsensitizers (e.g., 2-hydroxypropyl methacrylate) demonstrated peptide depletion values similar to those observed with the allergens suggesting good specificity for peptide reactivity assays. It is important to note that for a few test compounds (1,2-benzisothiazolin-3-one; 2-hydroxypropyl methacrylate; ethyl vanillin; and salicylic acid), one or two peptide depletion values are missing due to either an incompatibility with the solvent system or the test compounds coeluted with peptide in the HPLC analysis.

Prediction Models Based on Classification Tree Model Analysis

One requirement for using the peptide reactivity assay data for screening the skin sensitization potential of chemicals was to find a robust method to analyze and categorize the data. Another need was to determine if each of the five peptides were necessary for screening since the possibility of reducing the amount of work necessary to analyze each chemical would increase throughput and reduce the cost and amount of test material required for testing. To address these two needs, we chose to use a classification tree model approach which is a form of binary recursive partitioning that is used when observations need to be assigned to a category based on a number of predictor variables (Brieman *et al.*, 1983). Specifically, the classification tree approach used an algorithm to evaluate all of the peptide reactivity depletion data for each chemical in the context of its known LLNA potency category. Table 2 lists six prediction models that were developed based on use of all of the peptide data or limited to the use of specific peptide data (e.g., exclusion of GSH data). For each model generated, the model predictors used for evaluation of the chemical data set is given along with the model's accuracy and number of misclassifications. Cooper statistics were used to determine how well the model distinguished sensitizers (of any strength) and nonsensitizers. The Cooper statistics calculated accuracy based on chemicals predicted as sensitizers if they were categorized as having low, moderate, or high reactivity versus chemicals predicted as nonsensitizers if they were categorized as having minimal reactivity. It is clear from use of Cooper statistics analysis that model #1, which incorporates all of the peptides and their ratios, delivers the highest accuracy and fewest number of misclassifications. However, it is important to note that the delivered accuracy for the other models (#2– #6), which incorporate fewer peptides (e.g., model #5), is not that dissimilar to the more peptide comprehensive model #1. The similarity between the models suggest that for screening purposes it might be adequate to go with a model that uses fewer peptides and thus requires less material and less time for analysis. Comparison shows that the model #1 (sum of GSH; cysteine 1:10 and 1:50; and lysine 1:10 and 1:50) yields an accuracy of 94% and five misclassifications, whereas a model based only on cysteine 1:10 and lysine 1:50 yields a reasonable accuracy of 89% and nine misclassifications. Thus, we chose model #5 which includes only cysteine 1:10 and lysine 1:50 as predictors for analyzing further our peptide reactivity data.

Cysteine 1:10 and Lysine 1:50 Classification Decision Tree Model (Model #5)

The decision tree model that incorporates cysteine 1:10 and lysine 1:50 as predictors is presented in Figure 1. The model is based on making decisions on the average of peptide depletion data for cysteine 1:10 and lysine 1:50. As indicated in the methods, the classification decision tree model was developed using the peptide depletion data along with the LLNA potency data. By incorporating different cutoffs generated by the model, we have chosen to name the peptide depletion reactivity categories as minimal, low, moderate, and high reactivity. Generally, chemicals with moderate to high reactivity are associated with moderate to strong allergenicity, while those categorized as having minimal to low reactivity include weak and nonsensitizers (Table 3). However, it would be inappropriate to consider that a simple peptide reactivity assay would have the capability to predict a chemical's sensitization potency. It is believed that to accomplish this task, additional assay data will be needed to make an accurate prediction of a chemical's skin sensitization potential (Jowsey *et al.*, 2006). As far as the capability of using this model for classifying a chemical as a sensitizer or nonsensitizer, the Cooper statistics show that this model performs very well with an accuracy of 89% (Table 2 and Fig. 2). The high values for the sensitivity (88%), specificity (90%), positive predictivity (94%), and negative predictivity (81%) suggest that this peptide reactivity model would perform well as a screening assay, especially if used along with other physiochemical or biological data (Fig. 2). The nine chemicals that are misclassified include six sensitizers (α-hexylcinnamaldehyde, α-amylcinnamaldehyde, benzyl benzoate, 2,2,6,6-tetramethyl-3,5-heptanedione, oxalic acid, and nonanoyl chloride) and three nonsensitizers (2-acetylcyclohexanone, 2-hydroxypropyl methacrylate, and 1-bromobutane).

TABLE 2
Classification Tree Models Based on GSH, Cysteine, and Lysine Peptide Depletion Data

Model name	Model predictors used	Number of chemicals	Accuracy (%)	Number of misclassifications
Model #1	GSH, Cys (1:10 and 1:50), Lys (1:10 and 1:50)	78	94	5
Model #2	GSH, Cys (1:10 and 1:50)	82	88	10
Model #3	GSH, Cys (1:10), Lys (1:10)	78	91	7
Model #4	Cys (1:10 and 1:50), Lys (1:10 and 1:50)	78	91	8
Model #5	Cys (1:10), Lys (1:50)	81	89	9
Model #6	Cys (1:10)	82	89	9

DISCUSSION

Our understanding of the chemical and biological processes associated with skin sensitization and ACD has advanced significantly in recent years. This knowledge is providing the foundation for the development of numerous alternative methods for skin sensitization testing. One particular area of development has been to apply our knowledge of how chemical reactivity plays an important role in the initiation of skin sensitization response (reviewed in Lepoittevin *et al.*, 1998). Specifically, we (Gerberick *et al.*, 2004) as well as others (Aptula *et al.*, 2006; Divkovic *et al.*, 2005; Gerberick *et al.*, 2004a; Kato *et al.*, 2003; Natsch *et al.*, in press) have addressed the development of chemical reactivity screening methods for assessing the skin sensitization potential of chemicals.

It is believed that the formation of hapten-protein complexes is a prerequisite for the initiation of skin sensitization and which occurs prior to the processing of the complexes by antigen-presenting LC in the skin for the eventual presentation of the chemical to antigen-specific T cells. Chemical allergens (haptens) or their metabolites are small molecular weight compounds (generally less than 500 Da) with electrophilic properties. They are able to react with nucleophiles to form covalent bonds. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens. Lysine and cysteine are those most often cited but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can react with electrophiles (Ahlfors *et al.*, 2003; Dupuis and Benezra, 1982; Lepoittevin *et al.*, 1998). Thus, electrophilic allergens are believed to react with nucleophilic amino acids to form a stable covalent bond which is critical to the initiation of a skin sensitization response. However, it must be realized that other mechanisms for hapten interaction need to be considered as well (Divkovic, 2006).

Since reactivity is one key step in the induction of skin sensitization, we have been interested in pursuing whether measuring a chemical's reactivity could be used to develop a quantitative peptide-based reactivity assay that would have utility for screening a chemical's skin sensitization potency as defined in the LLNA. We evaluated 38 chemicals representing allergens of different potencies (weak to extreme) and non-sensitizers for their ability to react with GSH or three synthetic peptides containing either cysteine, lysine, or histidine (Gerberick *et al.*, 2004). The results demonstrated that a significant correlation exists between allergen potency and the depletion of GSH, lysine, and cysteine but not histidine. It is important to note that our intent in developing a peptide reactivity approach was not for the purpose of reproducing the physiological conditions of reactivity. For example, the lysine peptide assay must be run at pH 10.5 for optimal reactivity of the amine group. Moreover, we have chosen to focus on only two nucleophiles, lysine and cysteine, for use in developing a screening assay for determining a chemical's reactivity potential. Although lysine has been demonstrated to be an

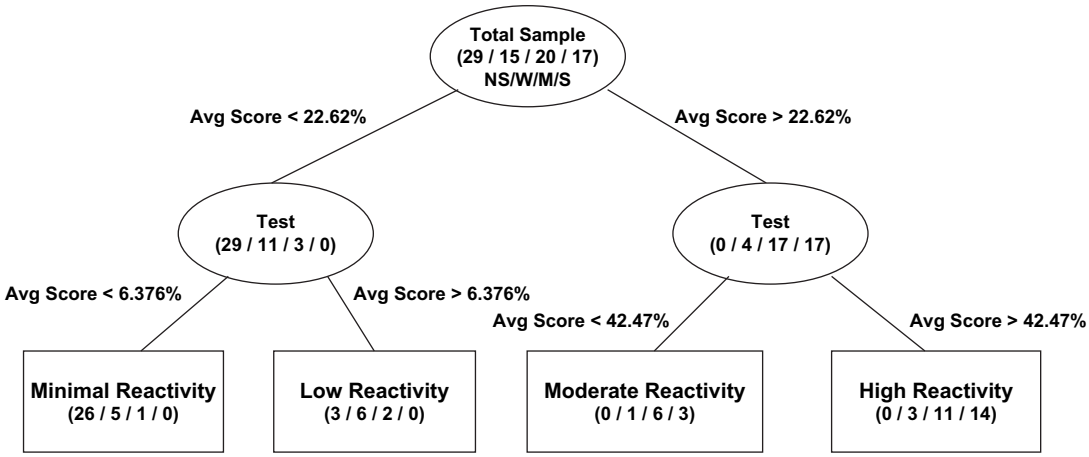

FIG. 1. Classification tree model based on the average of cysteine (1:10) and lysine (1:50) data.

TABLE 3
Comparison of Peptide Reactivity and Potency Data

Chemical name	EC3 value	LLNA category	Reactivity based on Cys (1:10) and Lys (1:50) data
Diphenylcyclopropenone	0.00030	Extreme	High
Oxazolone	0.0030	Extreme	High
Benzoyl peroxide	0.0040	Extreme	High
Kathon CG	0.0080	Extreme	High
Bandrowski's base	0.0080	Extreme	High
5-Chloro-2-methyl-4-isothiazolin-3-one	0.0090	Extreme	High
p-Benzoquinone	0.0099	Extreme	High
Tetrachlorosalicylanilide	0.040	Extreme	Moderate
2,4-Dinitrochlorobenzene	0.050	Extreme	High
Glutaraldehyde	0.10	Strong	High
Fluorescein isothiocynate	0.14	Strong	High
Phthalic anhydride	0.16	Strong	Moderate
Lauryl gallate	0.30	Strong	High
Propyl gallate	0.32	Strong	High
CD3	0.60	Strong	High
Trimellitic anhydride	0.60	Strong	Low
Formaldehyde	0.61	Strong	Moderate
Metol	0.80	Strong	High
2-Hydroxyethyl acrylate	1.4	Moderate	High
Glyoxal	1.4	Moderate	High
Vinyl pyridine	1.6	Moderate	Moderate
2-Mercaptobenzothiazole	1.7	Moderate	High
Nonanoyl chloride	1.8	Moderate	Minimal
2-Methyl-2H-isothiazol-3-one	1.9	Moderate	High
1,2-Benzisothiazolin-3-one	2.3	Moderate	High
Methyl-2-nonynoate	2.5	Moderate	High
Cinnamaldehyde	3.0	Moderate	High
Phenylacetaldehyde	3.0	Moderate	Moderate
Benzylideneacetone	3.7	Moderate	High
2,4-Heptadienal	4.0	Moderate	High
Squaric acid	4.3	Moderate	Moderate
Trans-2-hexanal	5.5	Moderate	High
Diethyl maleate	5.8	Moderate	High
2-Phenylpropionaldehyde	6.3	Moderate	Moderate
Perillaldehyde	8.1	Moderate	Moderate
Palmitoyl chloride	8.8	Moderate	Moderate
1-(4-Methoxyphenyl)-1-penten-3-one	9.3	Moderate	Low
a-Hexylcinnamaldehyde	11	Weak	Minimal
a-Amylcinnamaldehyde	11	Weak	Minimal
2,3-Butanedione	11	Weak	High
Farnesal	12	Weak	Low
Oxalic acid	15	Weak	Minimal
Benzyl benzoate	17	Weak	Minimal
4-Allylanisole	18	Weak	Low
Lilial	19	Weak	Low
Cyclamen aldehyde	22	Weak	Low
Imidazolidinyl urea	24	Weak	Moderate
5-Methyl-2,3-hexanedione	26	Weak	Low
2,2,6,6-Tetramethyl-3,5-heptanedione	27	Weak	Minimal
Ethyleneglycol dimethacrylate	28	Weak	High
Ethyl acrylate	28	Weak	High
Hydroxycitronellal	33	Weak	Low
Glycerol	NC[a]	NS[b]	Minimal
Hexane	NC	NS	Minimal
Diethyl phthalate	NC	NS	Minimal
Octanoic acid	NC	NS	Minimal
2-Hydroxypropyl methacrylate	NC	NS	Low
1-Butanol	NC	NS	Minimal
4-Hydroxybenzoic acid	NC	NS	Minimal
6-Methyl coumarin	NC	NS	Minimal
Methyl salicylate	NC	NS	Minimal
Chlorobenzene	NC	NS	Minimal
Lactic acid	NC	NS	Minimal
1-Bromobutane	NC	NS	Low
2-Acetylcyclohexanone	NC	NS	Low
4-Methoxyacetophenone	NC	NS	Minimal
Ethylbenzoylacetate	NC	NS	Minimal
Ethyl vanillin	NC	NS	Minimal
Isopropanol	NC	NS	Minimal
Propylene glycol	NC	NS	Minimal
Sulfanilamide	NC	NS	Minimal
Isopropyl myristate	NC	NS	Minimal
Benzaldehyde	NC	NS	Minimal
Methylparaben	NC	NS	Minimal
Nonanoic acid	21 (False +)	NS	Minimal
Propyl paraben	NC	NS	Minimal
Rsorcinol	NC	NS	Minimal
Salicylic acid	NC	NS	—
Sulphanilic acid	NC	NS	Minimal
Vanillin	NC	NS	Minimal
Coumarin	NC	NS	Minimal
Vinylidene dichloride	NC	NS	Minimal

[a]Not calculated.
[b]Nonsensitizer.

important nucleophile for allergens such as sultones and methylisothiazolone derivaties (Alvarez-Sanchez *et al.*, 2003; Meschkat *et al.*, 2001) and cysteine for α,β-unsaturated allergens (Ahlfors *et al.*, 2003), it is probable that nucleophiles other than lysine and cysteine are critical for the initiation of a sensitization response (Divkovic, 2006; Divkovic *et al.*, 2005). Thus, our approach will yield minimal information on how a specific chemical reacts with protein *in vivo* but does provide a means of quantifying reactivity for the purpose of screening skin sensitization potential.

Using GSH as a cysteine-containing peptide and two synthetic hepapeptides, one with lysine and other with cysteine, we expanded our analysis of chemical reactivity from 38 to 82 chemicals. The chemicals represented in the data set comprise weak ($n = 15$), moderate ($n = 19$), strong and extreme sensitizers ($n = 18$), as well as nonsensitizing materials ($n = 30$), as based on potency categorization criteria that have been developed by a European Centre for Ecotoxicology and Toxicology of Chemicals Task Force (Kimber *et al.*, 2003).

		Predicted Classification (based on classification tree model)		
		Non-Sensitizer	Sensitizer	total
Chemical Classification[a]	Non-Sensitizer	**26**	**3**	**29**
	Sensitizer	**6**	**46**	**52**
	total	**32**	**49**	**81**

table statistics for the shadowed 2 x 2 table

sensitivity:	88%
specificity:	90%
positive predictivity:	94%
negative predictivity:	81%
accuracy:	**89%**

[a]Based primarily on LLNA data

FIG. 2. Cooper statistics (nonsensitizers vs. sensitizers) for cysteine (1:10) and lysine (1:50) prediction model.

The LLNA EC3 values listed in Table 3 show a range of potency from 0.0003% for the extreme allergen, diphenylcyclopropenone, to 33% for the weak allergen, hydroxycitronellal.

The results, as summarized in Table 3, demonstrate that an association between the degree of peptide reactivity (as measured by nonreacted peptide depletion) and sensitization potency is evident. To help with interpretation of the peptide depletion data, we examined the utility of using classification tree methodology for development of a prediction model. Classification tree methodology involves an algorithm to group data based on one or more predictors. In this particular case, we used the peptide depletion data for each of the peptides (predictors) to see which ones would be used to subgroup the data. Although we used LLNA potency data for each of the 82 compounds to build the model, we chose to use high, moderate, low, and minimal reactivity as the category names for groups determined by the model. To evaluate each of the models for their hazard identification ability, we considered any compound that was categorized as high, moderate, or low as a skin sensitizer and those categorized as minimal as nonsensitizers. Cooper statistics were used to determine how well the different models distinguished sensitizers from nonsensitizers. All of the models generated are listed in Table 2. Model #1, that incorporated all of the peptide depletion data for each of the peptides, demonstrated a prediction accuracy of 94%. In addition, this model yielded only five misclassifications. Although the performance of this model is outstanding, it has the limitation of requiring the use of five different peptides. Moreover, model #1 involves the use of the GSH assay which involves a multiple-step procedure which can be challenging to transfer to other laboratories (data not shown). Thus, we were interested to see if a robust model could be developed that did not incorporate GSH into the decision tree. Table 2 summarizes the classification models and lists for each model the accuracy and number of misclassifications obtained with the model. Although the accuracy values are lower for the ''simpler'' models, they still show a very good ability to distinguish sensitizers from nonsensitizers. The one model we think demonstrates a good compromise between requiring fewer peptide ratios for analysis and no GSH is model #5, which includes use of cysteine at 1:10 and lysine at 1:50. Model #5 has a prediction accuracy of 89% with nine misclassifications. Of the six sensitizers classified as nonsensitizers, five of them are weak sensitizers (e.g., a-hexylcinnamaldehyde, benzyl benzoate). Nonanoyl chloride, a moderate sensitizer, was classified as a nonsensitizer. For chemicals that are misclassified, it is important to consider the chemical's water solubility as related to compatibility with the assay conditions as well as the possibility that the chemical is a prohapten and might require bioactivation prior to it reacting with nucleophile-containing peptides. Moreover, in some instances it might be prudent to review the LLNA data used to categorize a chemical as a sensitizer or nonsensitizer. For example, oxalic acid is categorized as weak sensitizer in the LLNA but the chemical does not contain apparent alerts or does human data exist to classify it as a sensitizer. Interestingly, a few of the nonsensitizers identified as sensitizers are compounds believed to have reactive properties (e.g., 2-hydroxypropyl methacrylate, 1-bromobutane). Moreover, two anhydride compounds are identified with the use of the lysine 1:50 peptide which support the use of using both cysteine and lysine for screening unknown chemicals. In addition to providing good assistance for hazard identification, the cysteine 1:10 and lysine 1:50 model provides quantifying data on reactivity that has potential for use, along with other data, for predicting the skin sensitization potency of an unknown chemical. Consistent with what we have observed in the past (Gerberick *et al.*, 2004), the amount of peptide depletion corresponds closely with the allergenic potency of the compound. Generally, moderate, strong, and extreme sensitizers show moderate to high reactivity, while weak and nonsensitizers show minimal to low reactivity (Table 3).

Of course, one would not expect an extremely high correlation between reactivity and potency since other factors, such as skin penetration and immune recognition by T cells, are critical for the acquisition of skin sensitization. It is not possible to say which event is most critical and it is likely unique for each chemical (e.g., reactivity for one chemical versus bioavailability for another chemical). Thus, it is very important to point out that it is not expected that the peptide reactivity assay alone should have the ability to predict a compound's sensitization potential. It is believed that to replace the LLNA, a battery of assays will be needed to reproduce the complex chemistry and biology that are involved in the induction of skin sensitization. Jowsey *et al.* (2006) have described this need in a very informative way by showing how different assays could provide quantitative information on different aspects known about the mechanism of ACD. For example, the peptide reactivity assay could serve as a first tier screening assay and also provide in time information needed to complete a holistic assessment of a chemical's skin sensitization potential.

One potential challenge for developing alternative methods for skin sensitization testing is that it is well known that some chemical allergens are prohaptens and as such require biotransformation prior to initiating a skin sensitization response *in vivo* (Smith and Hotchkiss, 2001). The need for biotransformation has been demonstrated with many chemicals, such as the formation of benzoquinonediimine from azo hair dyes (Basketter and Goodwin, 1988), or orthoquinone from isoeugenol (Bertrand *et al.*, 1997). Based on the knowledge that some chemical allergens need to be biotransformed prior to reacting with proteins/peptides, it will be critical to incorporate a metabolism component to address these types of molecules. We are currently evaluating a peroxidase/peroxide oxidizing system for use in a modified peptide reactivity assay.

The goal of this work was to evaluate the use of chemical reactivity as a means for screening the skin sensitization potential of chemicals. A prediction model was developed using a classification tree approach which allowed ranking the reactivity as minimal, low, moderate, or high as well as for assessing skin sensitization hazard. The results presented show clearly that using a cysteine- and lysine-based peptide depletion, assay demonstrates a good, but not perfect, association between chemical reactivity and allergenic potency. Generally, moderate to extreme allergens demonstrate high peptide depletion whereas weak and nonsensitizers demonstrate significantly less peptide depletion. It is hoped that with additional information from other *in vitro* assays and modification of existing peptide reactivity assays (e.g., addition of metabolism component), this methodology will be even more helpful in reducing our reliance on animals for skin sensitization testing in the future.

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