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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · MARCH 2004

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Protein Binding and Metabolism Influence the Relative Skin Sensitization Potential of Cinnamic Compounds

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Received July 10, 2003

Skin protein modification (haptenation) is thought to be a key step in the manifestation of sensitization to low molecular mass chemicals (<500 g/mol). For sensitizing chemicals that are not protein reactive, it is hypothesised that metabolic activation can convert such chemicals into protein reactive toxins within the skin. *trans*-Cinnamaldehyde, α-amyl cinnamaldehyde, and *trans*-cinnamic alcohol are known sensitizers with differing potencies in man, where the former two are protein reactive and the latter is not. Here, we have used immunochemical methods to investigate the extent of protein−cinnamaldehyde binding in rat and human skin homogenates that have been incubated (for either 5, 15, 30, or 60 min) at 37 °C with cinnamaldehyde, α-amyl cinnamaldehyde (at concentrations of between 1 and 40 mM), and cinnamic alcohol (at higher concentrations of 200 or 400 mM). Cinnamaldehyde specific antiserum was raised specially. A broad range (in terms of molecular mass) of protein−cinnamaldehyde adducts was detected (as formed in a time- and concentration-dependent manner) in skin treated with cinnamaldehyde and cinnamic alcohol but not with α-amyl cinnamaldehyde. Mechanistic observations have been related to relative skin sensitization potential, as determined using the local lymph node assay (LLNA) as a biological read-out. The work presented here suggests that there is a common hapten involved in cinnamaldehyde and cinnamic alcohol sensitization and that metabolic activation (to cinnamaldehyde) is involved in the latter. Conversely, there does not appear to be a common hapten for cinnamaldehyde and α-amyl cinnamaldehyde. Such mechanistic work on protein modification is important in understanding the early mechanisms of skin sensitization. Such knowledge can then be used in order that effective and appropriate in vitro/in silico tools for predicting sensitization potential, with a high confidence, can be developed.

Introduction

trans-Cinnamaldehyde and *trans*-cinnamic alcohol are both components of cinnamon spice and are used widely in the food and fragrance industry (*1*, *2*). α-Alkyl-substituted cinnamaldehydes (e.g., amyl- and hexyl-cinnamaldehyde) are also used in the fragrance industry. All of these compounds act as skin sensitizers, but with differing potencies, following skin contact in humans (*3*, *4*). It is a long-standing hypothesis that in order for a small molecule to act as a skin sensitizer it must modify skin protein. In doing so, the compound would then act as a hapten by transforming a self-protein into an immunogenic entity (*5*−*7*). Cinnamaldehyde possesses two electrophilic groups, and in theory, it could react directly with proteins via two different mechanisms—Schiff base formation to lysine residues or Michael addition to sulfhydryls (Figure 1) (*8*). Cinnamic alcohol, a significantly weaker sensitizer, is not protein reactive per se but it has been hypothesised that the alcohol is activated metabolically to cinnamaldehyde, which could act as a common hapten in both cinnamic alcohol and cinnamaldehyde skin sensitization (*9*). α-Alkyl-substituted cinnamaldehydes are also electrophilic but are less potent sensitizers in man (*10*); it is unknown as to whether they cross-react with cinnamaldehyde in humans or react with skin proteins via the same mechanism(s) as cinnamaldehyde. However, it is possible that differences in protein binding affinity or mechanism and/or metabolism may influence the differential potencies of these compounds.

This study was primarily aimed at investigating the mechanistic hypothesis that cinnamaldehyde is a common hapten in skin sensitization to both cinnamaldehyde and cinnamic alcohol. We also report the relative potencies of cinnamaldehyde, cinnamic alcohol, and α-methyl-, α-butyl-, α-amyl-, and α-hexylcinnamaldehyde, as determined using the local lymph node assay (LLNA) (*11*). In conjunction, we have mechanistically probed cinnamic compound-treated rat and human skin homogenates in vitro, using immunochemical methods with cinnamaldehyde adduct specific polyclonal antisera in order to detect and monitor the formation of protein−cinnamaldehyde adducts over time. Metabolic inhibition studies, using 4-methylpyrazole as an alcohol dehydrogenase inhibitor,

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10.1021/tx0341456 CCC: $27.50
Published on Web 02/04/2004

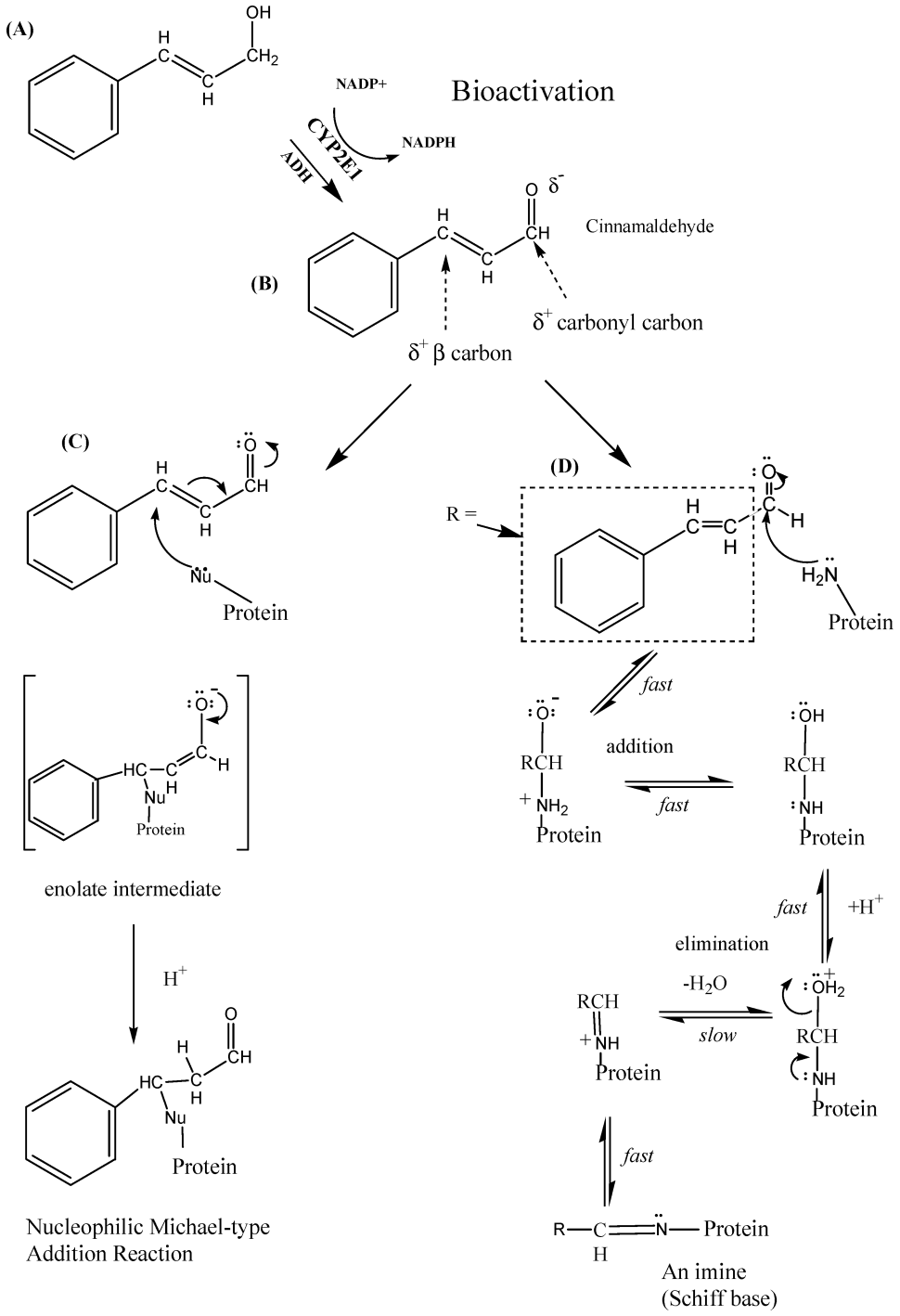

Figure 1. Possible mechanisms involved in the covalent binding of cinnamaldehyde to protein. (A) Cinnamyl alcohol, a prohapten, may be activated metabolically to cinnamaldehyde. (B) Illustrates the α,*β*-unsaturated carbon atoms of cinnamaldehyde. (C) Shows the nucleophilic attack on the *β*-unsaturated carbon via a 1,4-Michael addition reaction. (D) Schiff base formation between cinnamaldehyde and primary amine.

have been performed in cinnamic alcohol-treated skin in an attempt to inhibit the formation of protein−cinnamaldehyde adducts. These in vitro mechanistic data have been compared against the in vivo sensitization potency data to address the question of whether similar protein binding and metabolic processes are involved in the mechanism(s) of sensitization for cinnamic compounds.

Experimental Procedures

Materials. Cinnamaldehyde, cinnamic alcohol, cinnamic acid, hydroxycitronellal (HC), octanal, propenal, benzyl aldehyde, benzyl alcohol, ferulic acid, and 4-methylpyrazole were purchased from Sigma-Aldrich Inc. (Gillingham, U.K.). α-Methyl-, α-butyl-, α-amyl-, and α-hexylcinnamaldehydes (>98%) were kind gifts from Bush Boake Allen Ltd. (London, U.K.). Acrylamide (99.9% purity), bis(N,N′-methylene-bis-acrylamide), TEMED (N,N,N′,N′-tetramethyl-ethylenediamine), ammonium persulfate, SDS, Bromophenol Blue, molecular mass standards, and Coomassie Blue R-250 were all electrophoresis purity reagents purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts, U.K.) Casein was Hammersten grade from BDH ltd. (U.K.) Nitrocellulose (E) was 0.45 μm specification (Schleicher and Schuell, distributed in U.K. by Alderman and Co. Ltd., Kingston, Surrey). Alkaline phosphate (AP) and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG

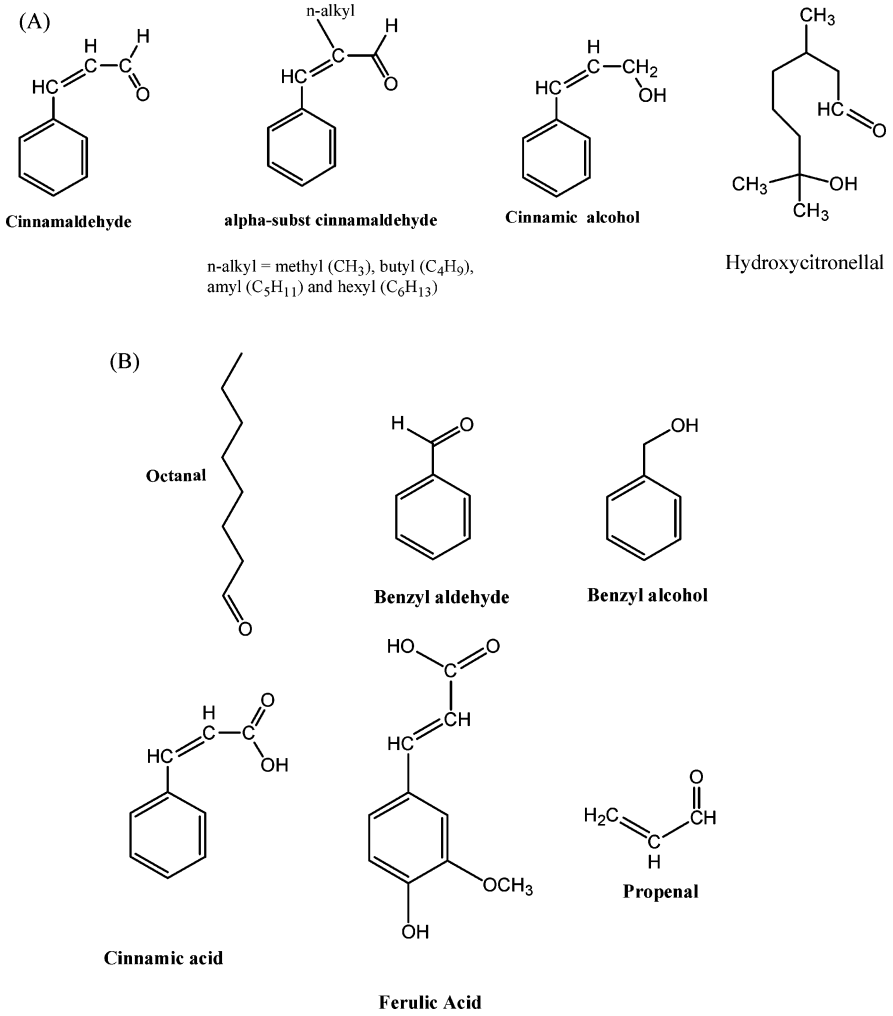

Figure 2. Structure of the chemicals used in hapten inhibition studies, which (A) inhibited binding of antibodies to the hapten−RSA conjugate and (B) did not inhibit antibody binding, as detected by ELISA.

were from Tago Immunodiagnostics Inc. (Burlingame, CA, distributed in U.K. by Serotec, Killington, Oxford). HRP-conjugated goat anti-rabbit IgG was also obtained from Dako Ltd. (High Wycombe, U.K.). Rabbit serum albumin (RSA), Freunds complete adjuvant, Freunds incomplete adjuvant, thimerosal, *o*-phenylenediamine, 30% hydrogen peroxide, sucrose, Tris-NaCl, and glycine were obtained from Sigma Chemical Co. (Poole, U.K.). Enhanced chemiluminescent and autoradiography films were obtained from Amersham (U.K.). All other chemicals were of the highest grade commercially available.

The purity of cinnamaldehyde and cinnamyl alcohol was analyzed using an isocratic reverse phase HPLC method, developed by Smith et al. (*12*). The purity and identity of the α-alkylcinnamaldehydes were verified by GC-MS using the method of Rastogi et al. (*13*).

LLNA. The doses and identities of chemicals tested in the LLNA are given in Figure 2 and Table 1. The protocol used followed the standard methodology described previously (*11* and in accordance with OECD test guideline 429). Groups of four CBA/Ca mice (7−12 weeks of age, Harlan Olac, U.K.) were treated with 25 μL of test material or with an equal volume of the vehicle (4:1 v/v acetone:olive oil) alone on the dorsum of both ears. Treatment was performed once daily for 3 consecutive days. Five days following the initiation of exposure, all mice were injected via the tail vein with 250 μL of PBS containing 20 μCi of tritiated thymidine (2 Ci $mmol^{-1}$) (Amersham International, Amersham, U.K.). Mice were sacrificed 5 h later, and the draining lymph nodes were excised and pooled for each experimental group. A single cell suspension of lymph node cells was prepared by mechanical disaggregation. The lymph node cell suspension was washed twice in an excess of PBS and then precipitated with 5% trichloroacetic acid (TCA) at 4 °C for 18 h. Pellets were resuspended in TCA, and the incorporation of tritiated thymidine was measured by β-scintillation counting. A substance was regarded as a skin sensitizer if at any test concentration the proliferation in treated lymph nodes was 3-fold or greater than that in the concurrent vehicle-treated controls (test/control cellular proliferation is termed the "stimulation index". In addition, the concentration of the chemical required to produce a stimulation index of 3, the EC3 value, was calculated as described previously (*14*) to provide a quantitative index of the relative sensitizing potency of chemicals tested).

Table 1. EC3 Values as Derived from the LLNA Data for Cinnamic Compounds Given as Molar and Percent, Representing the Concentrations above Which the Molecule Is Considered Sensitizing (*14*)

	EC3 (M)	EC3 (%)
cinnamaldehyde	0.10	1.3
α-methylcinnamaldehyde	0.30	4.5
α-butylcinnamaldehyde	0.58	11.2
α-amylcinnamaldehyde	0.52	10.6
α-hexylcinnamaldehyde	0.55	12.0
cinnamic alcohol	1.5	20.1

Synthesis of Cinnamaldehyde−RSA Conjugates. A 1 M stock solution of cinnamaldehyde was prepared in 10% methanolic PBS. A protein conjugate was prepared by incubating 40 mg of RSA (30 μM) in 20 mL of sodium phosphate buffer (pH 7.4) with 60 mg of cinnamaldehyde (20 mM final concentration)

at room temperature overnight with stirring. The conjugate solution was dialyzed using 10 kDa cutoff dialysis tubing at 4 °C against three changes of sodium phosphate buffer, pH 7.4, over 3 days with constant stirring to remove excess or unconjugated cinnamaldehyde. The protein solution was lyophilized, and the dry solid cinnamaldehdye−RSA conjugate was stored at 4 °C.

Determination of Free and Conjugated Amino Groups. To determine whether cinnamaldehyde had covalently modified lysine groups in RSA, the number of free lysine residues in RSA vs the cinnamaldehyde−RSA conjugate preparation were quantified using a modification of the method described by Habeeb (*15*). 2,4,6-Trinitrobenzyl sulfonic acid (TNBS or picryl sulfonic acid) reacts with free amino groups to give trinitrophenyl derivatives whose absorbance can be measured at 335 nm. One milliliter of equivalent test protein (either RSA or cinnamaldehyde−RSA conjugate) solutions were prepared using 10 mM Tris, HCl (0.1% SDS), pH 8.0, in 15 mL screw top glass tubes. One milliliter of 4% $NaHCO_3$, pH 8.5, was added and vortexed. A further 1.0 mL of TNBS (0.1%) was added, vortexed, and incubated at 40 °C for 2 h in a shaking water bath. The reaction was stopped by the addition of 1 mL of SDS (10%) followed by 0.5 mL of HCl, and the absorbance was read at 335 nm using quartz cuvettes (Fischer Scentific Laboratories, Leicestershire). Quantification was achieved by use of standard curves prepared from glycine where 1 mol of glycine is equivalent to 1 mol of free amino group.

Preparation of Antiserum. Two female New Zealand white rabbits (designated rabbits 1 or 2), obtained from Froxfield Farms (U.K.), body weight ∼2.5−3 kg, were immunized with the cinnamaldehyde−RSA conjugate, following a preimmunizaton bleed (20 mL) taken from the ear vein. The rabbits were injected subcutaneously across 6−8 sites on the back, with 1 mg of protein conjugate emulsified in 1 mL of filtered PBS, 0.15 M NaCl, 10 mM potassium phosphate, pH 7.4, in 1 mL of Freunds complete adjuvant. Two weeks following primary inoculation, 30 mL of blood was collected from the ear vein into glass tubes. At 3 weeks, the rabbits were boosted with 250 μg of protein conjugate in an emulsion prepared using Freunds incomplete adjuvant, and 30 mL of blood was collected from the ear vein 2 weeks after the boost. Boost and bleeding were repeated a further two times, and after the fifth boost, the blood was drawn from the ear vein and the animal was exanguinated. Rabbits were anaesthetized by administration of 0.2 mL/kg i.v. mixture of 1 part Hypnorm (2 mg/mL etomidate (Janssen Pharmaceuticals) and 2 parts water. Blood was obtained by cardiac puncture, allowed to clot for 1 h at room temperature in glass tubes, and cooled at 4 °C for 2 days to allow the clot to retract. Serum was carefully drawn off using a pipet and centrifuged at 10 000*g* for 20 min to leave a clear serum, aliquoted in 2 mL "O" screw cap vials, frozen, and stored at −70 °C.

ELISA. ELISA methodology was used to verify the affinity and specificity of the antisera for protein−cinnamaldehyde adducts. Aliquots (200 μL) of the test antigens, either cinnamaldehyde−RSA conjugate, RSA, human serum albumin (HSA), or HC−RSA conjugate (the latter as prepared previously (*16*) was diluted to 20 μg protein/mL in 50 mM sodium phosphate buffer, pH 5.0, and incubated overnight at 4 °C in 96 well microtiter plates (Immulon 4, Dynatch Labs)). Plates were washed with a wash buffer of 10 mM Tris, 0.15 M NaCl, 0.5 mM thimerosal, and casein 0.05%, pH 7.4, using an automated plate washer. Primary antiserum (200 μL of a 1:100 dilution in PBS) was added to each well and incubated for 3 h at room temperature. Plates were washed with wash buffer (four cycles), and the 200 μL of secondary antibody (goat anti-rabbit IgG, HRP conjugate, from Serotec Ltd., U.K.), at a dilution of 1:1000 in PBS, was added to the wells. After incubation for a further 2 h at room temperature, the plates were washed with four cycles of wash buffer followed by two cycles of PBS. Color development was started by the addition of 200 μL/well of *o*-phenylenediamine solution (0.4 mg/mL in 24 mM citrate, 50 nM sodium phosphate buffer, pH 5.0, containing 0.4 μL/mL of 30% H_2O_2) and was stopped after 8−10 min) by addition of 50 μL/well of 4 M H_2SO_4. Finally, the absorbance at 490 nm (A_{490}) of each well was determined using an automated plate reader, Titertek multiskan PLUS MKII, from Flow Laboratories Ltd. (U.K.).

Hapten Inhibition Studies. ELISA hapten inhibition studies were performed using a range of carefully selected potentially inhibitory chemicals (Figure 2). In these studies, the primary antisera were preincubated for 30 min, at room temperature, with a 1 mM concentration of the inhibitor compounds. Where necessary, the inhibitors were dissolved in methanol before addition to the diluted antisera. An identical ELISA procedure to that described above was performed using cinnamaldehyde−RSA as the test antigen bound to the plate and the preincubated sera.

Skin Homogenate Preparation. 1. Fresh Fischer Rat 344 Skin Homogenate Preparation. Fresh Fischer 344 rat skin, taken from the shaved dorsal region (10 g), was homogenized according to the method previously described by Cheung et al (*17*), except that buffer A (aqueous physiological receptor fluid (0.025 M Hepes, Hank's balanced salts solution (9.8 g/L), 4 mM sodium bicarbonate, 50 mg/L gentamycin, pH 7.4)) was used. Briefly, the skin was cleaned of all connective tissue and fat and either kept whole or the epidermis was removed using an overnight incubation at room temperature in Dispase. The skin pieces were chopped in 2 mL of either buffer A or B using a scalpel and scissors into ∼1 mm pieces and placed into 20 mL scintillation vials. Ten milliliters of buffer was added, and the skin pieces were homogenized on ice for 3.5 min in 30 s bursts using an Ultra-Turrax T25, probe S25N-10G, (Janke & Kunkel, IKA Labortechnik, Germany) at 24 000 rpm. The sample was filtered through nylon gauze (N140, Lockertex, Warrington, U.K.) with a pore size 240 μm to remove any large debris. The supernatant was dounced 10 times in a glass homogenizer to further disrupt the homogenate and form a single cell suspension. The skin homogenate cell suspensions were stored at −20 °C until required.

2. Fresh Human Skin Homogenate Preparation. Human breast tissue was obtained from a 45 year old female Caucasian who underwent surgery at St. Mary's Hospital, London. Directly following excision, the tissue was placed in cold 0.9% saline solution and then used immediately. Human skin samples were obtained with ethical approval from the Local Research Ethics Committee of St. Mary's Hospital. Fresh human skin was homogenized as above using buffer B (25% methanolic 50 mM sodium citrate, pH 5.5).

In Vitro Cinnamic Compound Incubation with Skin Homogenates. A 200 μL amount of skin homogenate samples was diluted in the respective buffer to a final volume of 500 μL in 1.5 mL cryo-vials. Samples were treated with 1−20 μM (final concentration) cinnamaldehyde or 1−400 μM (final concentration) cinnamic alcohol or 1−400 μM (final concentration) α-amyl cinnamaldehyde. Control samples were untreated. Each fraction was incubated for 5, 10, 15, or 60 min or 18 h at 37°. In a similar but separate experiment, skin homogenates were pretreated with varying concentrations of 4-methyl pyrazole (4-MP) (20−200 mM final concentration), for 15 min at 37 °C, prior to treating with cinnamic alcohol. Rat skin homogenate samples were also heat treated for 15 min at 95 °C and allowed to cool for 15 min prior to adding cinnamic alcohol (400 μM final concentration). The homogenates were snap frozen in liquid nitrogen to stop the reaction and stored at −80 °C. The total protein content of all fractions was determined using the standard method by Lowry et al. (*18*).

Western Blot Analysis. Samples for Western blot analyses were prepared for electrophoresis in Laemmli's sample buffer containing 6 mg/mL dithiothreitol. SDS−PAGE was performed using BIO-RAD Mini Protean II gel system, with 4% acrylamide stacking and 10% acrylamide resolving minigels (run until the dye front reached the bottom of the gel) using standard techniques (*19*). A 20 μL amount containing 5 μg of total protein was loaded per sample. Transfer of the separated proteins from

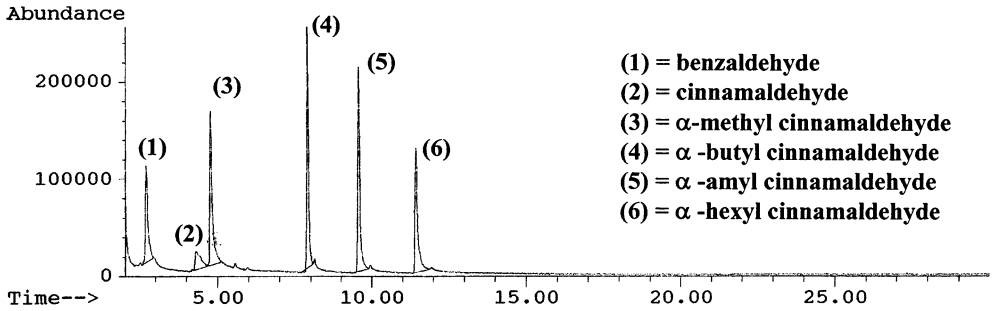

Figure 3. GC separation of benzaldehyde, cinnamaldehyde, and α-alkyl-substituted cinnamaldehydes using the method of Rastogi, et al. (1995). Compound identity was verified by coupled GC-MS.

the gel to nitrocellulose membrane was performed at 150 V for 1 h at 4 °C. Nonspecific binding sites on the membrane were blocked overnight in casein blocking buffer at 4 °C in 10 mM Tris-HCl buffer, pH 7.6, containing 0.25% (w/v) casein, 0.15 M NaCl, and 0.5 mM thimerosal. The membranes were developed for immunoreactivity using cinnamaldehyde−RSA antiserum. All antibodies were diluted to 1:10 000 in 10 mM Tris-HCl buffer, pH 7.6, containing 0.05% w/v casein, 0.15 M NaCl, and 0.5 mM thimerosal. Antibody binding was detected using a goat anti-rabbit IgG to HRP (Appligene-Oncor Lifescreen, U.K.), diluted 1:40 000, visualized using enhanced chemiluminescence, and recorded on hyperfilm and developed in Kodak D19 developer and Kodak Rapid Fix.

Immunohistochemistry. Full thickness human skin samples were cleaned of fat and connective tissue and placed in a Petri dish containing buffer A, on a wire mesh, and cinnamaldehyde and or cinnamic alcohol (10 μL neat) were carefully added to the surface of the skin and left overnight at 37 °C. Skin samples were cut into 0.5 cm^2 and placed in 4% paraformaldehyde (buffered with sodium phosphate to pH 7.4) and fixed overnight at 4 °C before processing into paraffin wax embedded blocks. Sections (5 μm) were cut and placed onto poly-L-lysine coated slides. Processing and sectioning were performed as a service by the Department of Histopathology at St. Mary's Hospital, London.

All immunohistochemistry procedures were performed at room temperature. Skin sections were deparafinized in xylene and rehydrated in three ethanol washes of decreasing percentage (100 to 70%). Endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide:TBS (Tris-buffered saline), and nonspecific sites were blocked by 25% normal goat serum: TBS. Sections were then probed with anti CAld−RSA antisera or nonimmune sera and diluted with TBS (1:20 000). Antibody binding was detected using a goat anti-rabbit IgG conjugated to biotin (DAKO, U.K.) (diluted in TBS to 1:500) with subsequent bound biotin detected using a HRP streptavidin conjugate (Vector Laboratories, Peterborough, U.K.) (diluted in TBS to 1:200). Bound peroxidase activity was observed by developing with diaminobenzidine conjugate (Vector Laboratories). Sections were counterstained with Gill's haematoxylin and visualized with light microscopy.

Results

Purity of Cinnamic Compounds. Cinnamaldehyde was 96% pure (3.26% cinnamic acid and 0.71% cinnamic alcohol impurities). Cinnamic alcohol was 96.5% pure (0.51% cinnamaldehyde and the remaining 3.02% impurities were unidentifiable by HPLC and of a polar nature). All of the α-alkyl cinnamaldehydes could be separated and unequivocally identified using GC-MS. The results of GC separation are shown in Figure 3. All of the latter materials were >98% pure.

LLNA Results. The stimulation indices from the LLNA for all cinnamic compounds tested (plus benzaldehyde as a nonsensitizing negative control) are presented in Figure 4. EC3 values are given in Table 1. Of all six compounds, cinnamaldehyde was the strongest sensitizer (i.e., had the lowest EC3 value). The α-alkylcinnamaldehydes were only sensitizing at the top dose tested of 25% in acetone:olive oil. At this dose, there appeared to be a semiquantitative structure−activity relationship, i.e., the longer the alkyl chain, the less sensitizing the cinnamic analogue as determined by a decreasing stimulation index (Figure 4). In terms of EC3 values, the order of sensitization potency was cinnamaldehyde > α-methylcinnamaldehyde > α-butylcinnamaldehyde = α-amylcinnamaldehyde = α-hexylcinnamaldehyde > cinnamic alcohol. Cinnamic alcohol was the weakest sensitizer and exhibited an approximately 15-fold higher EC3 value than cinnamaldehyde.

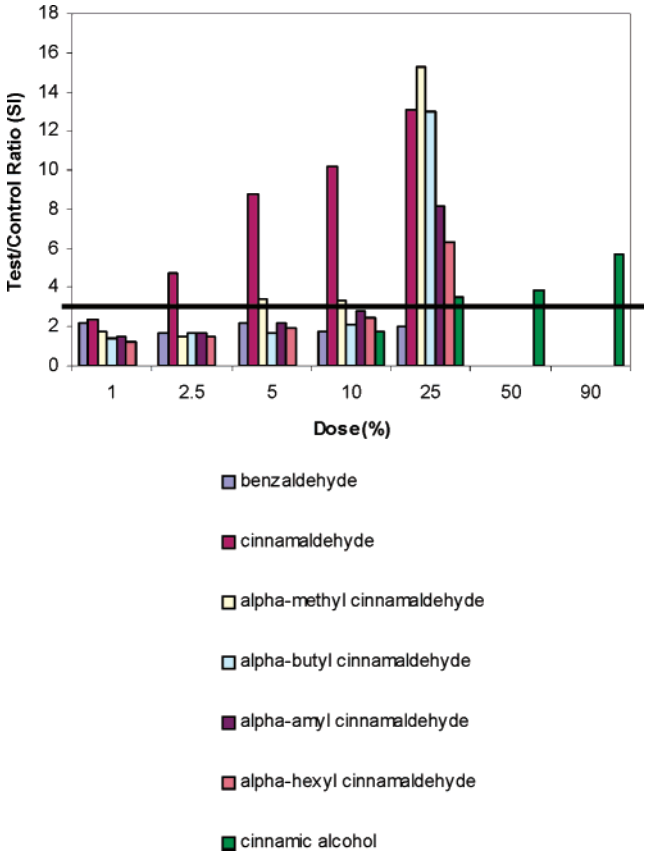

Figure 4. Stimulation indices derived from LLNAs performed at a range of doses for cinnamaldehyde (CAld), cinnamic alcohol (CAlc), and α-methyl-, α-butyl-, α-amyl-, and α-hexyl-cinnamaldehyde (MetCAld, ButCAld, AmylCAld, and HexCAld, respectively). All compounds were dosed in a 4:1 acetone:olive oil vehicle. The bold line represents a test/control ratio of 3 above which the compound is considered as a sensitizer. Benzaldehyde was used as a known nonsensitizing control.

In Vitro Conjugation of Cinnamaldehyde to RSA. Upon conjugation with cinnamaldehyde, RSA was converted from a white solid material to a lyophilized yellow solid. An estimate of free amine group modification (e.g., lysine and arginine side chains) by cinnamaldehyde in the CAld−RSA conjugate was obtained by TNBS titration, using unconjugated RSA and glycine as reference materials.

Native RSA was found to contain 102 amino groups that were free to react with TNBS using this method; the SwissProt sequence database indicates that RSA contains 127 amine groups. Analysis of the cinnamaldehyde−RSA conjugate indicated that 22 free amines per molecule of RSA had been modified by cinnamaldehyde. Of the 102 measured free amine groups, this implies that ∼22% of total free amino groups had been modified by this approach. The accepted practice for raising antisera to protein conjugates is based on the assumption that the highest antibody (IgG) titer is obtained with a hapten density of 15−30 molecules per carrier (*20*). Hence, the level of cinnamaldehyde conjugation to RSA was considered appropriate for inoculating rabbits to raise antisera.

ELISA Analyses of Antisera Specificity and Affinity. ELISA revealed that antisera from both rabbits 1 and 2 contained high titers of antibodies that recognized the cinnamaldehyde−RSA conjugate antigen against which the sera were raised. Bleeds taken from rabbit 1 at weeks 8 and 11 following primary inoculation showed the strongest recognition for the antigen. To test specificity, other test materials, i.e., a HC−RSA conjugate and unconjugated RSA or HSA, were incubated with sera. Wells were also incubated with PBS or left empty as positive controls. The antisera exhibited low degrees of recognition to these other materials. However, the overall response to cinnamaldehyde−RSA was clearly stronger (Figure 5). The antisera did not react with PBS alone. The preimmunization bleed did not show any recognition to any of the test materials. However, there was some recognition to unconjugated RSA and HSA as the test antigen in ELISA, which could be eliminated by preincubation of the sera with RSA or HSA.

Detection of Protein−Cinnamaldehyde Adducts in Cinnamic Compound-Treated Skin Homogenates. Protein adducts that were formed in skin homogenates treated with either cinnamaldehyde or cinnamic alcohol were identified by Western blotting, using anti-cinnamaldehyde−RSA antisera from rabbit 1 (either week 8 or 11 bleeds) (Figures 6A and 7). No recognition of any proteins could be effected using preimmune sera in Western blots in agreement with ELISA. Similarly, no adducts were detected in the control (untreated skin fraction) and the vehicle controls, methanol and 4-MP. However, a ∼66 kDa adduct was detected in control human skin fractions, which was eliminated when the immune sera were preincubated with HSA (Figure 6Aii). The molecular mass of this protein band and inhibition with HSA suggest that the serum was cross-reacting with HSA derived from the skin.

1. Cinnamaldehyde-Treated Skin Homogenates. A broad spectrum of skin proteins in both human (Figure 6A) and rat (Figure 7) skin homogenates appeared to be modified by cinnamaldehyde, covering the molecular mass range between 30 and 200 kDa. No similar recognition of proteins in untreated control samples was observed (except for low level detection of a protein at ∼66 kDa at an incubation time of 1 h in the rat skin homogenates, likely to be unmodified RSA) (Figure 7). This exemplified the low degree of nonspecificity of the antisera for unmodified skin proteins.

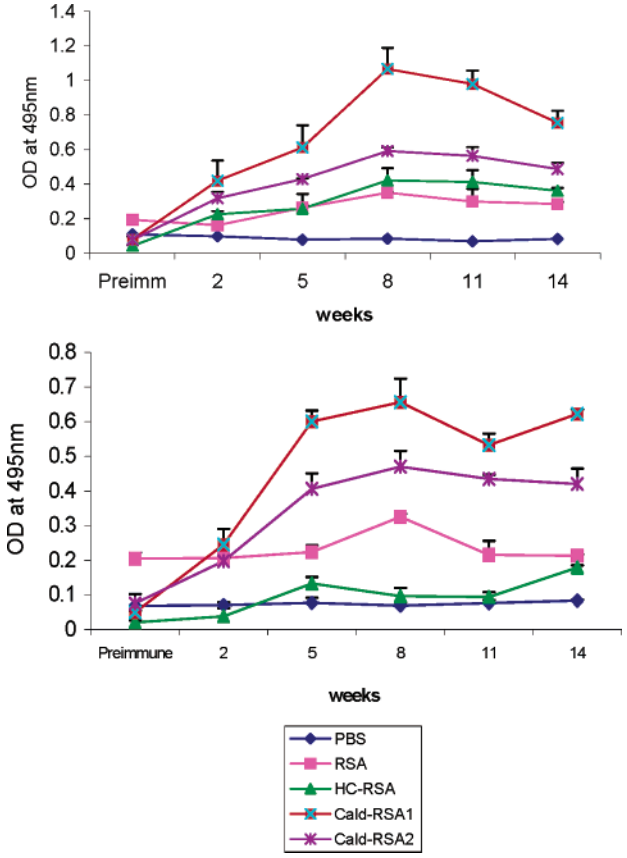

Figure 5. Characterization of antisera specificity using ELISA rabbit antisera from two cinnamaldehyde−RSA immunized rabbits (A and B) was tested. Serum was collected at 2, 4, 8, 11, and 14 weeks postimmunization. Maximal reactivity against the test antigen (RSA−cinnamaldehyde conjugate), as measured using optical density measurements at 495 nm, was seen at 8 weeks following the primary inoculation in both rabbits. The specificity of the antisera was also tested against a HC−RSA conjugate and native RSA. Primary antisera preferentially recognized both batches of RSA−cinnamaldehyde antigen antiserum (at a serum dilution 1:50). Each data point is the mean of $n = 3$ determinations.

The breadth of formation of cinnamaldehyde−protein adducts increased with increasing both cinnamic compound concentration and incubation time (Figures 6A and 7). At the lowest concentration tested (1 or 2 mM), only one or a few proteins appeared to be modified at ∼66 kDa or less and at any time point. Upon increasing the concentration of cinnamaldehyde to 10 or 20 mM and incubating for 30 min or more, a much broader spectrum of proteins appeared to be modified (Figures 6A and 7). In both rat and human skin homogenates, adducts first appeared with the highest cinnamaldehyde concentration tested (20 mM) after 5 or 15 min of incubation (Figure 7). These protein adducts were of a high molecular mass, possibly skin keratins derived from the stratum corneum or epidermis.

2. Cinnamic Alcohol- and α-Amylcinnamaldehyde-Treated Skin Homogenates. Protein−cinnamaldehyde adducts were identified in samples of cinnamic alcohol-treated skin but could not be identified in samples of α-amylcinnamaldehyde-treated skin (Figure 6A). No protein adducts could be identified using low concentrations of cinnamic alcohol (1−10 mM) and incubation

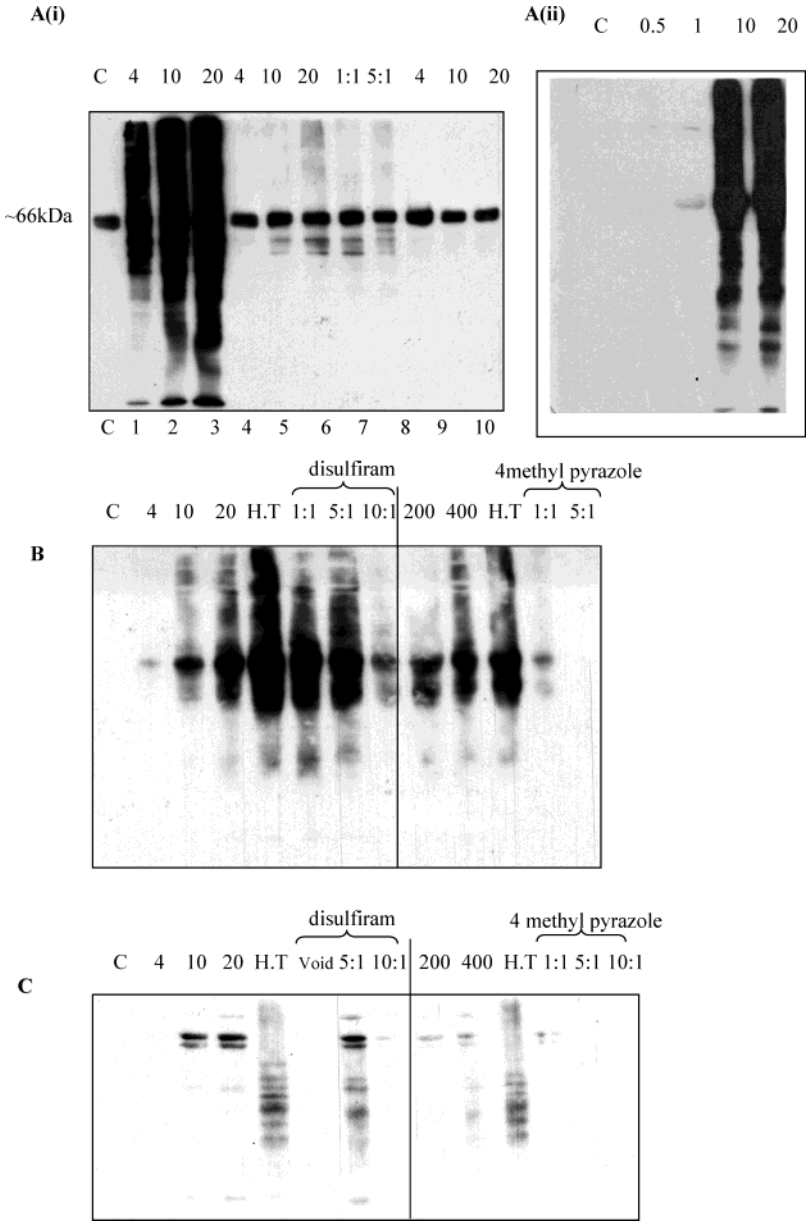

Figure 6. Immunoblot detection of cinnamaldehyde modified protein adducts in cinnamic allergen-treated skin homogenate fractions. Western blot analysis of cinnamaldehyde modified protein (Ai) in fresh human skin homogenates treated with 4, 10, and 20 μM Cald (lanes 1−3), CAlc (lanes 4−6), or α-amyl-CAld (lanes 9−11); lanes 7 and 8 = ADH inhibitory work using 20 and 200 μM 4-MP with 20 μM cinnamyl alcohol (all 5 μg in 20 μL of total protein loaded). In human skin, the antisera were seen to cross-react significantly with a ~66 kDa protein. When the sera were pretreated with HSA (66 kDa protein), this band disappeared (Aii)—the pretreated sera were used to probe the same batch of treated human skin as seen in panel Ai but with different cinnamaldehyde concentrations (0.5−20 μM). (B) Rat skin epidermis and (C) rat skin dermis, incubated with cinnamic allergens. C = control untreated skin fraction; lanes 4, 10, and 20 = the dose in μM of cinnamaldehyde; lanes 200 and 400 = the dose in μM of cinnamyl alcohol; HT = heat treated for 15 min prior to adding 400 mM cinnamyl alcohol; lanes 1:1, 5:1, and 10:1 = is the ratio expressed in μM of disulfiram (d) or 4-MP (m) to 20 and 400 μM cinnamaldehyde and cinnamyl alcohol, respectively; 5 min exposure of film.

times of 1 h or less. However, using a 20 mM cinnamic alcohol concentration and a 30 min incubation time, a single band at ~66 kDa was observed (Figure 6A). Increasing the concentration to 40−200 mM and at incubation times of 30 min or more, a broader spectrum of higher molecular mass adducts was identified.

To inhibit the formation of these protein adducts (presumably formed via metabolic activation, by alcohol dehyrogenase (ADH)/cytochrome P450 2E1, of cinnamic alcohol to cinnamaldehyde, which then binds to protein). the skin samples were preincubated with 4-MP, an alcohol dehydrogenase/cytochrome P450 inhibitor. Concentration ratios of 1:1 or 5:1 4-MP:cinnamic alcohol inhibited the formation of protein adducts, and a ratio of 10:1 eliminated the formation of adducts using a concentration of 20 mM CAlc and an incubation time of 30 min or 1 h (Figures 6A and 7). Heat treating the fractions at 95 °C for 15 min and then cooling the homogenate to room temperature prior to adding cinnamic alcohol, the aim being to inactivate enzymes, did not affect the formation of protein adducts.

Upon separating rat epidermis from dermis, the profile of adducts was very different between these subtissue types (Figure 6B,C). Many more epidermal proteins appeared to be modified than dermal proteins.

Hapten Inhibition Studies. Hapten inhibition studies, using anti-cinnamaldehyde−RSA sera preincubated with RSA or the compounds in Figure 2, were undertaken

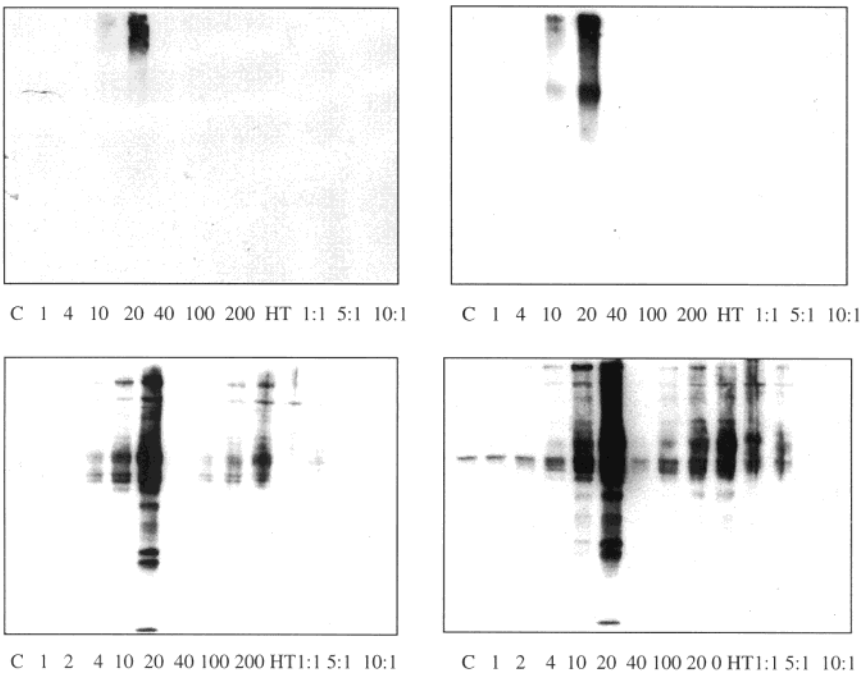

Figure 7. Immunoblot detection of cinnamaldehyde modified protein adducts in cinnamic allergen-treated rat skin homogenate fractions. Western blot analysis of cinnamaldehyde modified protein adducts in fresh rat whole skin homogenates at (A) 5, (B) 10, (C) 15, and (D) 60 min. Nitrocellulose membranes were probed with anti CAld−RSA antisera (diluted 1:15 000), secondary goat anti-rabbit HRP (dilution 1/20K). C = control untreated skin fraction; lanes 1, 4, and 10 = the dose in μM of cinnamaldehyde; lanes 40, 100, and 200 = the dose in μM of cinnamyl alcohol; HT = heat treated for 15 min prior to adding 400 mM cinnamyl alcohol; lanes 1:1, 5:1, and 10:1 = is the ratio expressed in μM of 4-MP to 400 μM cinnamyl alcohol.

to probe the mechanisms and compound selectivity of antiserum recognition.

1. Western Blotting. Preincubation of the antisera with RSA inhibited the recognition of the 66 kDa band (presumably HSA) in human skin samples but did not inhibit protein adduct recognition. As expected, significant inhibition was observed when antisera were incubated with the cinnamaldehyde modified RSA, the conjugate to which the antisera was raised against (blots not shown).

2. ELISA Studies. ELISAs revealed that anti-cinnamaldehyde−RSA serum recognition of the cinnamaldehyde−RSA antigen could be inhibited when the serum was preincubated with 1 mM concentrations of certain compounds (Table 2). If the sera were preincubated with a 1 mM concentration of the antigen to which it was raised, this effected ~41% inhibition of antigen recognition in ELISAs. Preincubation of the immune sera with 1 mM of cinnamaldehyde or any of the α-alkyl-substituted cinnamaldehydes resulted in significant and similar inhibition of antigen recognition (Table 2). HC, a nonbenzylic skin sensitizing aldehyde, also moderately inhibited antigen recognition by 14%. Cinnamic alcohol weakly inhibited antigen recognition (by 3%). None of the other compounds inhibited antigen recognition.

Table 2. ELISA Inhibition Studies: 1 mM Concentrations of the Following Compounds Were Used to Preincubate the Antisera[a]

1 mM compound	inhibition of recognition as detected by ELISA (%)
cinnamaldehyde−RSA conjugate	41.15 ± 7.99
native RSA	2.97 ± 0.46**
cinnamaldehyde	27.76 ± 7.75 ns
α-methyl cinnamaldehyde	24.39 ± 4.27*
α-butyl cinnamaldehyde	35.26 ± 2.57 ns
α-amyl cinnamaldehyde	26.93 ± 10.12 ns
α-hexyl cinnamaldehyde	27.32 ± 6.04 ns
cinnamic alcohol	3.09 ± 0.89*
HC	13.38 ± 1.92*
octanal	no inhibition
benzyl aldehyde	no inhibition
benzyl alcohol	no inhibition
cinnamic acid	no inhibition
ferulic acid	no inhibition
2-propenal	no inhibition

[a] The percentage inhibition of recognition of immobilized cinnamaldehyde−RSA antigen is given. *$P < 0.05$, ** $P < 0.01$, ns = not significant. The statistical test applied was a two-tailed and type 2 *t*-test; data array as compared to the mean values obtained from cinnamaldehyde−RSA inhibitor.

Discussion

Since the 1930s, the key hypothesis that describes an early mechanistic event of the skin sensitization process is that low molecular mass chemicals must react with or modify skin proteins to form higher molecular mass immunogenic hapten−protein conjugates (*5*, *7*, *21*). However, there is little empirical confirmation of this hypothesis. This study reports for the first time the widespread covalent modification of skin proteins by skin sensitizers. This work also corroborates previous work (*22*−*24*) in that model, intact, purified proteins (e.g., albumin) and model nucleophiles can be modified covalently in vitro by skin sensitizers. For example, ^{13}C NMR techniques have been used to study hapten reactivity to model nucleophiles (e.g., propanethiolate and imidazole), as well as HSA, to characterize binding mechanisms of hex-1-ene, hexane-1,3-sultones, and isothiazolinone preservatives (*22*−*24*). Probing mechanisms of sensitization in vitro using model proteins not only allow theoretical predictions to be verified but also allow the broader spectrum of reactivity and novel mechanisms to be revealed.

In this work, we have examined the protein reactivity profiles of the sensitizers cinnamaldehyde, amylcinnamaldehyde, and cinnamic alcohol using immunobiology techniques. These chemicals are not only found naturally in cinnamon but also are present in the European standard "Fragrance Mix" allergen. While direct mechanisms of protein reactivity for cinnamaldehyde and amylcinnamaldehyde can be postulated, cinnamic alcohol is a sensitizer but does not possess protein reactive functional groups. Hence, it has been postulated that cinnamic alcohol must be activated to cinnamaldehyde in order to become reactive (*9*). If this hypothesis is true, common protein−hapten adducts should be picked up in both cinnamaldehyde- and cinnamic alcohol-treated skin using cinnamaldehyde specific antisera. This work has revealed this to be the case, and a diverse spectrum of cinnamaldehyde-modified proteins was observed in rat and human skin upon treatment in vitro with both cinnamaldehyde and cinnamic alcohol. Hence, this confirms that cinnamaldehyde is a common hapten in cinnamic alcohol sensitization.

How is cinnamic alcohol converted to cinnamaldehyde within the skin? The metabolic inhibition data presented here, using the alcohol dehydrogenase inhibitor 4-methylpyrazole, supports the hypothesis of metabolic activation of cinnamic alcohol into cinnamaldehyde via oxidoreductase pathways (*9*). It is known that the skin is a metabolic organ (*7*), but the direct involvement of metabolism in effecting the early stages of sensitization has yet to be proven. The work here goes some way to providing evidence that activating metabolism can play a part in the ability of apparently nonreactive small molecule sensitizers to modify skin proteins via their metabolites.

Skin protein−cinnamaldehyde modification was not observed in skin homogenates treated with α-amylcinnamaldehyde, suggesting that the α-alkyl derivative is not converted to cinnamaldehyde within the skin. However, it is theoretically possible that α-alkylcinnamaldehydes can modify proteins via a similar mechanism(s) to cinnamaldehyde, but such modification would not be expected to lead to any cross-reactivity with cinnamaldehyde itself, as reaction would not lead to a common hapten. It would also be expected that the reaction center may be less electrophilic in α-alkyl-substituted derivatives due to the influence of the inductive effect of the alkyl substituent on the adjacent carbon atom. Hence, there are expected differences in protein-binding mechanisms between cinnamaldehyde and alkylcinnamaldehydes that may confer different skin sensitization properties. As observed in the LLNA results shown here, α-alkylcinnamaldehydes are much less potent sensitizers than cinnamaldehyde but more potent than cinnamic alcohol. This observation together with the lack of cinnamaldehyde adducts in amylcinnamaldehyde-treated skin suggest that there are direct mechanisms of protein binding for alkylcinnamaldehydes that do not require metabolism and do not yield cinnamaldehyde as a common hapten.

An interesting observation in Western blotting experiments is that a 10−20-fold higher concentration of cinnamic alcohol than cinnamaldehyde is required to give a similar extent of skin protein modification. While this may be a coincidental observation, given the complexity of the immunological response, it is interesting to relate this possibly rate-limiting metabolic difference to the 15-fold difference in sensitization potency in the LLNA between these two compounds. If such a good correlation between extent of protein binding and LLNA potency could be confirmed, the principle could form the basis of an in vitro assay to estimate sensitization hazard and potency.

These studies have also revealed that the extent of protein binding is dependent upon both concentration of compound and incubation time. Such an observation supports the premise that exposure parameters (skin exposure time and dose delivery to target skin proteins) are important in the clinical manifestation of allergic contact dermatitis. The earliest time point where protein modifications were observed was at 5 min incubation with 20 mM cinnamaldehyde. The apparent binding at this time point was to high molecular mass proteins only, possibly keratins, derived from the stratum corneum and epidermis. With longer incubation times and higher concentrations, the spectrum of protein modification became more diverse to include lower molecular mass proteins. This is consistent with what is known about the function of skin, in that the keratin-rich stratum corneum acts as a defensive barrier to exogenous compounds and a higher binding affinity to keratins could act as a protective mechanism.

It can be expected that there would be different affinities of protein binding/modification at different molecular sites in different types of skin proteins in different compartments of the skin tissue. While there exists a high degree of specificity in relation to chemical-induced allergy, from the data presented here, it appears that many different skin proteins can be modified. Whether all of the skin proteins that are modified in vitro are immunologically relevant remains to be determined. The exact amino acid residues that are modified in vivo may be dependent upon the chemical microenvironments within protein macromolecules that allow the relevant chemistries to take place, either sterically or kinetically. From immunohistochemistry studies using cinnamaldehyde-treated rat skin sections (data not presented), protein modification was seen to be diffuse and not localized in any particular skin tissues/cells. Although many proteins appear to be modified in all regions of the skin, we can still expect that there are certain amino acid residues where binding is more favored than others. Determining the exact nature of the amino acids modified is the topic of further work using mass spectometry analyses (*29*).

To probe the antisera recognition elements that led to specific recognition of cinnamaldehyde-derived hapten, inhibition studies were performed using chemicals that are related in structure to cinnamaldehyde. As anticipated, preincubation of the antisera and the antigen to which the antisera was raised yielded significant inhibition of antigen recognition in ELISA. Preincubation of the antisera with cinnamaldehyde, the α-alkylcinnamaldehydes, HC, and to a lesser degree cinnamic alcohol also yielded inhibition of antigen recognition. All of the inhibitory compounds (except cinnamic alcohol) are electrophilic, and it is hypothesized that the observed inhibition is effected by the chemicals that could covalently modify the recognition elements of the antibody proteins contained within the antisera and thus prevent antigen recognition. If noncovalent association of the chemical with proteins in the antisera were causative of the observed antigen recognition inhibition in ELISA, then

some of the other nonelectrophilic chemicals should also have effected inhibition.

The data presented here indicate that a skin sensitizer can bind to many skin proteins. However, it is likely that binding to certain amino acid residues in macromolecules can be selective and dependent upon the existence of chemically favorable microenvironments (e.g., local pH or the orientation of the chemical in a sterically favored pocket adjacent to the reaction site). Protein binding is expected to be a first key step in the complex immunological events of sensitization. In vitro cell-based systems that incorporate the key mechanistic events for sensitization, including relevant intrinsic protein-binding chemistries and activating metabolism, could be powerful alternative indicators of sensitization hazard for the future. Identification of the major protein targets in skin at the molecular level will be important knowledge to gain in this respect (*29*). It remains to be determined whether relative sensitization potency could also be estimated in vitro, but it is likely that knowledge of chemical reactivity alone will not be sufficient for this purpose. Such knowledge will need to be refined by an understanding of the immunologically relevant protein substrate, and this is the subject of further work.

Acknowledgment. We acknowledge the financial support of the Research Institute of Fragrance Materials (RIFM) Inc. and the European Cosmetic, Toiletry and Perfumery Association (COLIPA).

References

(1) Letizia, C. S., Cocchiara, J., Wellington, G. A., Funk, C., Isola, D. A., and Api, A. M. (1999a) Safety Evaluation of Cinnamaldehyde. *Report to the Research Institute for Fragrance Material*.

(2) Letizia, C. S., Cocchiara, J., Wellington, G. A., Funk, C., Isola, D. A., and Api, A. M. (1999b) Safety Evaluation of Cinnamic Alcohol. *Report to the Research Institute for Fragrance Material*.

(3) Johansen, J. D., Andersen, K. E., Rastogi, S. C., and Menne, T. (1996a) Threshold responses in cinnamic-aldehyde-sensitive subjects: results and methodological aspects. *Contact Dermatitis 34* (3), 165−171.

(4) Johansen, J. D., Rastogi, S. C., and Menne, T. (1996b) Exposure to selected fragrance materials. A case study of fragrance-mix-positive eczema patients. *Contact Dermatitis 34*, 106−110.

(5) Landsteiner, K., and Jacobs, J. (1936) Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med. 64*, 643−655.

(6) Dupuis, G., and Benezra, C. (1982) *Allergic Contact Dermatitis to Simple Chemicals: A Molecular Approach*, Marcel Dekker Inc., New York and Basel.

(7) Smith, C. K., and Hotchkiss, S. A. M. (2001) *Allergic Contact Dermatitis: Chemical and Metabolic Mechanisms*, Taylor & Francis Ltd., London, U.K.

(8) Majeti, V. A., and Suskind, R. R. (1977) Mechanism of cinnamaldehyde sensitization. *Contact Dermatitis 3*, 16−18.

(9) Basketter, D. A. (1992) Skin sensitization to cinnamic alcohol: The role of skin metabolism. *Acta Dermatol. Venereol. 72*, 264−265.

(10) Basketter, D. A., Blaikie, L., Dearman, R. J., Kimber, I., Ryan, C. A., Gerberick, G. F., Harvey, P., Evans, P., White, I. R., and Rycroft, R. J. G. (2000) Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis 42*, 344−348.

(11) Kimber, I., and Basketter, D. A. (1992) The murine local lymph node assay; collaborative studies and new directions: A commentary. *Food Chem. Toxicol. 30*, 165−169.

(12) Smith, C. K., Cheung, C., Elahi, E. N., and Hotchkiss, S. A. M. (2001): A high-performance liquid chromatography method for the quantification of nonradiolabeled cinnamic compounds in analytes derived from human skin absorption and metabolism experiments. *J. Chromatogr. Biomed. Sci. Appl. 758* (2), 249−264.

(13) Rastogi, S. C., Lepoittevin, J. P., Johansen, J. D., Frosch, P. J., Menne, T., Bruze, M., Dreier, B., Andersen, K. E., and White, I. R. (1998) Fragrances and other materials in deodorants: search for potentially sensitizing molecules using combined GC-MS and structure activity relationship (SAR) analysis. *Contact Dermatitis 39* (6), 293−303.

(14) Basketter, D. A., Lea, L., Cooper, K., Dickens, A., Briggs, D., Pate, I., Dearman, R. J., and Kimber, I. (1999) A comparison of statistical approaches to derivation of EC3 values from local lymph node assay dose responses. *J. Appl. Toxicol. 19*, 261−266.

(15) Habeeb, A. F. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem 14* (3), 328−336.

(16) Tonge, R. P. (1995), The cutaneous disposition of the sensitising chemicals hydroxycitronellal and dinitrochlorobenzene. Thesis, Pharmacology and Toxicology, St. Mary's Hospital, London, University of London.

(17) Cheung, C., Smith, C. K., Hoog, J.-O., and Hotchkiss, S. A. M. (1999) Expression and localisation of human alcohol and aldehyde dehydrogenase enzymes in skin. *Biochem. Biophys. Res. Commun. 261*, 100−107.

(18) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin reagent. *J. Biol. Chem. 193*, 265−275.

(19) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbour Laboratory Press, Plainview, New York.

(20) Malaitsev, V. V., and Azhipo, O. Y. (1993) Influence of epitope density on the immunogenic properties of hapten-protein conjugates. *Bull. Exp. Biol. Med. 115*, 726−728.

(21) Basketter, D. A., Dooms-Goossens, A., Karlberg, A.-T., and Lepoittevin, J.-P. (1995) The chemistry of contact allergy: Why is a molecule allergenic? *Contact Dermatitis 32*, 65−73.

(22) Meschkat, E., Barratt, M. D., and Lepoittevin, J. (2001a) Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 1. Synthesis and studies on the reactivity toward model nucleophiles of the (13)C-labeled skin sensitizers hex-1-ene- and hexane-1,3-sultones. *Chem. Res. Toxicol. 14*, 110−117.

(23) Meschkat, E., Barratt, M. D., and Lepoittevin, J. (2001b) Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 2. NMR studies of the covalent binding of the (13)c-labeled skin sensitizers 2-[13C]- and 3-[13C]hex-1-ene- and 3-[13C]hexane-1,3-sultones to human serum albumin. *Chem. Res. Toxicol. 14*, 118−126.

(24) Alvarez-Sanchez, R., Basketter, D., Pease, C., and Lepoittevin, J.-P. (2003) Studies of Chemical Selectivity of Hapten, Reactivity, and Skin Sensitization Potency. 3. Synthesis and Studies on the Reactivity towards Model Nucleophiles of the 13C-Labeled Skin Sensitizers, 5-Chloro-2-methylisothiazol-3-one (MCI) and 2-Methylisothiazol-3-one. *Chem. Res. Toxicol. 16* (5), 627−636.

(25) Roberts, D. W., and Benezra, C. (1993) Quantitative structure−activity relationships for skin sensitisation potential of urushiol analogues. *Contact Dermatitis 29*, 78−83.

(26) Roberts, D. W., York, M., and Basketter, D. A. (1999) Structure−activity relationships in the murine local lymph node assay for skin sensitisation: alpha, beta-diketones. *Contact Dermatitis 41*, 14−17.

(27) Patlewicz, G. Y., Basketter, D. A., Smith, C. K., Hotchkiss, S. A., and Roberts, D. W. (2001) Skin-sensitization structure−activity relationships for aldehydes. *Contact Dermatitis 44*, 331−336.

(28) Patlewicz, G. Y., Wright, Z. M., Basketter, D. A., Smith-Pease, C. K., Lepoittevin, J. P., and Arnau, E. G. (2002) Structure−activity relationships for selected fragrance allergens. *Contact Dermatitis 47* (4), 219−226.

(29) Divkovic, M., Basketter, D., Gilmour, N., Dell, A., Morris, H., and Smith Pease, C. K. (2003) Protein-Hapten Binding: Challenges and Limitations. *J. Toxicol. Cutaneous Ocul. Toxicol. 22* (1, 2), 87−99.

TX0341456