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Induction Elicitation of Contact Sensitivity in Mice due to Prohapten

L Khan [a], R J Schmidt [b] S N Gilani [a], N Shafi [a] and K D Ahmad [a]*

[a]*Medicinal Botanic Centre, PCSIR Laboratories, Peshawar-25100, Pakistan*

[b]*Welsh School of Pharmacy, UWCC, PO Box 13, Cardiff CF1 3XF, U K*

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The prohaptenic status of abietic acid, methylabietate, 3-methylcholanthrene (3-Mc), 3-pentadecylphenol (3-PDP), and 5-pentadecylresorcinol (5-PDR), was confirmed by partition coefficient studies and conjugation reactions with amino acids. The sensitizing effects observed on mouse skin showed activation of prohapten into hapten during the induction and elicitation of the contact sensitization reactions.

Key words: Prohapten, Partition coefficient, Amino acid conjugation, Contact sensitization.

Introduction

Most of the chemicals implicated in Allergic Contact Dermatitis (ACD) are low molecular weight substances which can not by themselves provoke an allergic reaction. They require to combine to larger molecules (Proteins) inside the skin to become antigenic. In such conditions the chemical acts as a hapten and the larger molecule as a carrier (Landsteiner and Jacobs 1935) Not all chemicals implicated in ACD are haptens. If the range of the chemicals known to induce ACD is studied, it becomes obvious that most of them do not have the required activity for binding with proteins. It has now been accepted that chemicals which do not appear to have reactivity with proteins are appropriately known as prohapten (Dupuis and Benezra 1982) that is they require activation before they can act as haptens. In the present studies, the preimmunological and immunological events of prohapten activation were studied. Prohaptens included in these investigations were Abietic acid, Methylabietate, 3-Methylcholanthrene (3-Mc), 3-pentadecylphenol (3-PDP) and 5-pentadecylresorcinol (5-PDR). Hapten chosen for the studies were 2,4-dini-trochlorobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB).

Materials and Methods

Materials: Abietic acid was isolated from Colophony (WW) by the method of Harris and Sanderson (1948) Methylabietate was prepared by the interaction of abietic acid and oxalyl chloride. 3-methylcholanthrene (3-Mc), was obtained from Aldrich Chemical Co Ltd Gillingham U.K. Commercial grade samples of 3-pentadecylphenol (3-PDP) 95% and 5-pentadecylresorcinol (5-PDR) 85% were further purified by recrystallisation from petroleum spirit (60-80°C) and toluene. 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitorfluorobenzene (DNFB) were obtained from Aldrich Chemical Co. Ltd. For partition coefficient studies, *n*-octanol analar grade and for conjugation reactions amino acids cysteine, cystine, glycine, histidine, methionine, proline, tryptophan and tyrosine were obtained from BDH Chemicals Ltd. Poole UK. For the induction of contact sensitization, WSP male mice were used. Skin fold thickness measurements were carried out using an Oditest gauge (Kroplin Ltd. UK).

Partition coefficient: Calibration curves for abietic acid, methylabietate, 3-Mc, 3-PDP, 5-PDR, DNCB and DNFB were constructed from solutions in *n*-octanol and 0.1 M Phosphate buffer pH 7.4. Partition coefficients were determined by the shake flask method (Leo *et al* 1971).The partitioning system comprised of *n*-octanol and 0.1 M Phosphate buffer pH 7.4. Before partitioning each phase was saturated with the other by addition of 5% v/v. The solutions were shaken for two hours and then allowed to stand overnight. Test materials prohapten 20 mg and hapten 10 mg were withdrawn, each was suspended in 10 ml pre-equilibrated *n*-octanal and added to 90 ml or 50ml phosphate buffer pH 7.4. The solutions shaken for 24 h at room temperature. After layers separation, the absorption of aqueous solutions were monitored on a double beam UV spectrophotometer using phosphate buffer as a blank. The quantities of the test materials partitioned into the aqueous phase were calculated from the regression equations. The apparent partition coefficient was calculated as ratio of the concentration in the two phases at equilibrium.

Conjugation: Equimolar amounts of prohapten, hapten and amino acids were used. Prohapten and hapten were dissolved in 1 ml acetone and transferred to test tubes containing individual amino acids suspended in 5 ml 0.1 M phosphate buffer pH 7.4 The test tubes holding both solutions were stoppered and incubated at room temperature for 24 h. After incubation, the contents of the test tubes were concentrated and fractionated into aqueous and chloroform extracts. Both

*Author for correspondence

aqueous and organic extracts were evaluated on TLC. 10 µl of the aqueous extracts were spotted on 0.25 mm thick cellulose layers and developed in a solvent system consisting of water/methanol/acetic acid 70:20:10. Afterwards the plates were air dried, sprayed with ninhydrin reagent and heated for 5 min in an oven maintained at 100°C. Similarly chloroform extracts were spotted on 0.25 mm thick silica gel G 60 PF 254 + 366 layers and developed in a solvent system consisting of petroleum spirit (60-80°C)/chloroform/methanol 20:10:1. The plates were air dried and evaluated under UV light.

Contact sensitization: Male mice, 10 Weeks old were divided into eight groups. For each chemical 10 control and 10 test animals were used. Before treatment each mouse was shaved, an area of about 2x2 cm over the back was shaved. The concentrations of the test materials employed during the induction phase were chosen on the basis of mouse ear irritancy assay (Evans and Schmidt 1979). Acetone was used as a vehicle. During the induction phase, the individual in each cage of 10 mice were treated epicutaneously on every other day with acetone (200 µl), abietic acid (15 mg/200 µl), methylabietate (15 mg/200 µl), 3-PDP (10 mg/200 µl), 5-PDR (15 mg/200 µl), 3-Mc (2 mg/200 µl), DNCB (2 mg/200 µl), and DNFB (2 mg/200 µl). A visual assessment of the skin reactions was made on days 10 and 6. Reactions were categorised as being either positive or negative in conjunction with skin fold thickness measurements of the treated sites using an Oditest gauge. Animals were then allowed to recover until days 24 and 21 when they were challenged. For challenge reaction, the same doses were used as were employed during the induction phase. On days 26 and 23 a visual assessment of the skin reaction at the test site was made and skin fold thickness measurements were again determined.

Results and Discussion

The apparent partition coefficients of the chemicals are listed in Table 1. In some cases the absorbance of the test materials in the aqueous layer approached the lower limit of detection, presumably due to their negligible partition. In those cases the *n*-octanol layer was evaluated for the determination of partition coefficients, but the calculated quantities turned out very close to the original amounts taken for partitioning. Obviously in these cases partition coefficients could not be accurately determined. Certain physical and chemical properties are thought to be important for penetration of a chemical into the skin. Best penetration can be achieved with smaller molecules (mol. wt. 400 dalton) and chemicals which are easily miscible with the aqueous and the lipid solvents (Bronaugh and Maibach 1983) As evident from the result in Table 1, abietic acid exhibited miscibility with the aqueous phase and therefore will display some degree of penetration into the skin during sensitization reaction. On the contrary methyl abietate lacked association with the aqueous layer and hence its penetration across the skin layers can not be predicted. Similarly 3-Mc showed negligible degree of miscibility with the aqueous phase. 3-PDP and 5-PDR possess hydrophilic functional groups but both have shown negligible association with the aqueous phase. Non alkylated phenol and resorcinol have been shown to be miscible with aqueous solutions. The reported partition coefficients of phenol is 1.46 and resorcinol 0.80 (Fujita *et al* 1964). The observed partition coefficients of 3-PDP and 5-PDR suggest that the most obvious factor that has masked their miscibility with aqueous solution is the alkyl side chains. It has been reported that alkyl side chains, when long enough, tend to coil up around the molecule in solutions. This normally results in the formation of molecular oil droplets. In contrast to prohapten the hapten DNCB and DNFB exhibited a higher degree of miscibility with both aqueous and lipid layers. This result suggests that hapten will penetrate more readily into the skin during epicutaneous application. The observed partition coefficients of the chemicals further suggest that strong and weak contact allergenicity in experimental animals, due to prohapten and hapten, may arise either from an ability to penetrate the skin quickly or from an inability to penetrate the skin. As the prohaptens showed a higher degree of lipid solubility, therefore their penetration across the stratum corneum will be by a slow diffusion process. Perhaps they may be penetrating into the skin more readily in the later stages of exposure because it has been demonstrated (Schalla and Schaefer 1982) that prolonged treatments of the skin can disturb the integrity of the horny layer and also weaken its barrier properties.

The results of the conjugation of prohapten, and hapten with amino acid are listed in Table 2. During the conjugation reaction, conditions were chosen to simulate as far as possible the aqueous environment of the body with respect to pH and temperature. Spots on the chromatograms other than those for the starting materials were taken to indicate that adduct formation had occurred. In no case was a chloroform soluble adduct observed and it was clear that in most but not all cases, unchanged compounds were present. In cases where the starting materials were no longer present, water soluble adducts could be detected. In most cases DNCB and DNFB produced adducts with the amino acids, Abietic acid and its methyl ester have failed to interact with the amino acids, which suggests that they may not be implicated in contact sensitization reactions unless transformed into reactive species. The results are consistent with the earlier finding (Karlberg *et al* 1985) that abietic acid is not a contact allergen because it cannot form an electrophilic species to conjugate with proteins. However since abietic acid and its derivatives are easily oxidised compounds, it is possible

that oxidised derivatives may be the hapten. 3-Mc has also not produced conjugates with the amino acids but it has been reported that in the presence of mixed function oxidase, 3 Mc is metabolised into a number of reactive species such as epoxides, phenol and quinone etc. These species have strong affinities for proteins and other macromolecules inside the skin (Levin *et al* 1979). It is as a result of metabolic activation that 3-Mc is believed to act as a potent carcinogen. 3-PDP and 5-PDR are the constituents of a complex mixture of closely related compounds known as urushiols (Landsteiner and Jacob 1936) Although the components of urushiols are potent contact allergens, they do not appear to have reactivity with proteins and amino acids. 3-PDP and 5-PDR have also demonstrated negligible interaction with amino acids. The results are consistent with the earlier findings, that 5-PDR lacks binding characteristics with proteins and therefore not capable of behaving as a hapten. Perhaps for a similar reason other investigators have concluded that due to the lack of quinone formation, 5-PDR was not capable of effecting *in vitro* lymphocyte blastogenesis which was induced and elicited by compounds capable of transforming into quinones (Byers *et al* 1979). The question arises what activates 3-PDP and 5-PDR into immunogens. Perhaps a free radical species might be responsible for their sensitizing activity (Schmidt and Khan 1990).

The results of contact sensitization in mice as a result of exposure to test materials are listed in Table 3. The evaluation of skin reactions was also made on the basis of skin fold thickness measurements of the test site. In all control and treated groups of animals, skin fold thickness measurements were generally very consistent. As evident from the results the test materials proved as contact sensitizers. Abietic acid and methyl abietate showed mild skin reactions. The degree of sensitization was very low. Moreover there was not any significant difference in the skin fold measurements between the control and treated animals. The results obtained did not support the *in vivo* transformations of abietic acid or its ester into reactive metabolites by metabolising enzymes. Perhaps both chemicals decompose on the skin (Khan 1988) Mice treated with 5-PDR showed positive skin reactions. The degree of sensitization and skin fold thickness measurements were considerable. The results support the activation of 5-PDR into a reactive species during the induction of contact sensitization.

Table 1

Apparent partition coefficients of the test materials

Test materials /quantities	Absorbance of the aqueous phase C_2	Amount of the test materials partitioned in the aqueous phase CW(mg).	Amount of the test material left in the organic phase CO (mg).	Apparent partition coefficients P=CO/CW	Log_{10} P
Abietic acid (20 mg)	0.1	0.12/90 ml	19.87/ 10 ml	1529.23	3.18
Methyl abietate (20 mg)	-	-	-	-	-
3-MC (20 mg)	-	-	-	-	-
3-PDP (20 mg)	-	-	-	-	-
5-PDR (20 mg)	-	-	-	-	-
DNCB (10 mg)	0.25	0.25/ 50 ml	9.74/10 ml	190.31	2.28
DNFB (10 mg)	0.36	1.16/ 50 ml	8.84/ 10 ml	38.10	1.58

* No. of replicates, 3.

Table 2

Conjugation of prohapten, hapten with amino acids

Amino acids	Test materials						
	Abietic acid	Methylabietate	3-MC	3-PDP	5-PDR	DNCB	DNFB
Cysteine	-	-	-	-	-	-	-
Cystine	-	-	-	-	-	-	-
Glycine	-	-	-	-	-	+	+
Histidine	-	-	-	-	-	+	+
Methionine	-	-	-	-	-	+	+
Proline	-	-	-	-	-	+	+
Tryptophan	-	-	-	-	-	-	-
Tyrosine	-	-	-	-	-	+	+

(+) TLC evidence of adduct formation; (-) No TLC evidence of adduct formation.

Table 3
Contact sensitization of mice

Test materials	No. of animals	Treatment on days with (a)	Induction phase Reaction read on days 10 and 6*: Skin reaction	Skin (C) thickness mm ± SD	Challenge reaction animals treated on day 24 and 21*: Reaction read on day 26 and 23*: Skin reaction	Skin (C) thickness mm ± SD
Abietic acid	10	1, 3, 5, 7, 9	3(+) 7(-)	0.9 ± 0.010	3(+)	0.9 ± 0.010
Methyl abietate	10	1, 3, 5, 7, 9	3(+) 7(-)	0.9 ± 0.010	3(+)	0.9 ± 0.010
5-PDR	10	1, 3, 5, 7, 9	7(+) 3(-)	1.4 ± 0.039	6(+) 1(-)	1.4 ± 0.039
3-PDP*	10	1, 3, 5	8(+) 2(-)	1.6 ± 0.026	6(+) 2(-)	1.6 ± 0.026
3-MC*	10	1, 3, 5	8(+) 2(-)	1.5 ± 0.046	7(+) 1(-)	1.5 ± 0.046
DNCB*	10	1, 3, 5	10(+)	3.2 ± 0.081	10(+)	3.0 ± 0.067
DNFB*	10	1, 3, 5	10(+)	3.5 ± 0.081	10(+)	3.0 ± 0.067
Acetone	10	1, 3, 5, 7, 9	70(-)	0.8 ± 0.006	70(-)	0.8 ± 0.006

a). Induction dose Abietic acid, Methyl abietate, 5-PDR, 15mg/200µl/; 3PDP 10mg/200µl 3MC, DNCB,DNFB 2mg/ 200µl/ animal/ application.
b). Challenge dose Abietic acid, methyl abietate, 5-PDR, 15mg/200µl; 3-PDP 10mg/200µl; 3-MC, DNCB, DNFB, 2mg/ 200µl.
c). Skin reaction. Sensitization reaction were categorised as being either positive or negative.
d). Skin thickness Skinfold thickness at site of reaction.

Animals exposed to 3-PDP showed distinct skin reactions. Even with smaller doses and limited applications per day, contact sensitivity was induced. The major feature of the skin reaction was that at the onset of sensitization, depletion of the skin occurred i.e., sensitized animals had developed alopecia, the shedding and thinning of hair were observed over the treated sites. The skin irritating effects of 3-PDP and 5-PDR has been reported in a study on the active ingredients of the oil of the cashew nut shell (Keil *et al* 1945; Stahl *et al* 1983) The test conducted on 150 individuals had demonstrated that 3-PDP, 5-PDR and anacardic acid cause rashes. Animals exposed to 3-Mc showed distinct skin reactions. These were marked by the presence of oedema and thickening of the test site. In addition, at the onset of sensitization, cracks appeared in the horny layer and depletion of the treated area occurred. 3-Mc lacks reactive groups that bind with proteins and other macromolecules inside the skin. However, it is a well known inducer of the mixed function oxidase enzymes and during metabolism transforms into a number of reactive metabolites which later bind with the skin proteins and other cellular constituents (Pohl *et al* 1976).

Animals treated with DNCB and DNFB showed positive skin reactions. The results are consistent with the earlier finding (Dupuis and Benezra 1982). Both chemicals are haptens and can easily bind with proteins to become antigenic.

Conclusion

The results presented, though only of a preliminary nature in mice, open up the possibility that prohapten can be activated in or on the skin into highly reactive species by various enzymatic and non-enzymatic processes. Therefore contact allergenicity may be the result of binding of reactive species to skin protein. Sensitizers possessing the ability to penetrate the skin and also bind with skin protein can be implicated in the induction and elicitation of contact sensitization reaction.

References

Bronaugh R L, Maibach H I 1983 *In vitro* percutaneous absorption In: *Dermatoxicology*, Marzulli F N & Maibach H I. Hemisphere Publishing Corp, Washington New York, London 2nd ed pp 117-129.

Byers V S N, Castagnoli Jr, Epstein W L 1979 *In vitro* studies of poison oak immunity. *J Clin Invest* **64** 1449-1456.

Dupuis G, Benezra C 1982 *Allergic Contact Dermatitis to Simple Chemicals. A Molecular Approach.* Marcel Dekker New York, Basel p 66.

Evans F J, Schmidt R J 1979 An assay procedure for the comparative irritancy testing of esters in the tiglane and daphnane series. *Inflammation* **3** 215-223.

Fujita T, Iwasa J, Hansch C 1964 A new substituent constant derived from partition coefficients. *J Am Chem Soc* **86** 5175-5180.

Harris G C, Sanderson T F 1948 Resin acids. An improved method of isolation of resin acids. *J Am Chem Soc* **70** 334-339.

Karlberg A T, Bergstedt E V A, Boman A, Bohlinder K, Liden C, Lars J, Nilsson G, Wahlberg J E 1985 Is abietic acid the allergenic component of colophony *Contact Dermatitis* **13** 209-215.

Keil H, Wassermann D, Dawson C R 1945 The relation of hypersensitiveness to poison ivy and cashew nut shell liquid. *Science* **102** 279-280.

Khan L 1988 A study of prohapten activation in contact allergy. Ph D thesis, Department of Pharmaceutical Chemistry, Welsh School of Pharmacy UWCC, Cardiff, UK.

Landstenier K, Jacobs J L 1935 Studies on the sensitization of animals with simple chemical compounds. *J Exp Med* **61** 643-656.

Landsteiner K, Jacobs J L 1936 Studies on the sensitization of animals with simple chemical compounds. *J Exp Med* **64** 625-639.

Leo A, Hansch C, Elkins D 1971 Partition coefficients and their uses. *Chem Rev* **71** 525-616.

Levin W, Buening M K, Wood A, Chang R, Thakker D, Jerina D, Conney A 1979 Tumorigenic activity of 3-methylcholanthrene metabolites on mouse skin and in new born mice. *Cancer Res* **39** 3549-3553.

Pohl R, Philpot R, Fouts J 1976 Cytochrome p-450 content and mixed function oxidase activity in microsomes isolated from mouse skin. *Drug Metab Dispos* **4** 442-450.

Schalla W, Schaefer H 1982 Mechanism of penetration of drugs into skin. In *Dermal and Transdermal Absorption*, eds Brandau R & Lippold B H. Wissenschaftliche Verlagsgesellchaft Gmb Stuttgart pp 41-72.

Schmidt R J, Khan L, Chung L Y 1990 Are free radicals and not quinone the haptenic species derived from urushiols and other allergenic mono and dihydric alkylbenzenes. *Arch Dermatol Res* **282** 56-64.

Stahl E, Keller K, Blinn C 1983 Cardanol, a skin irritant in pink pepper. *Planta Medica* **48** 5-9.