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SDS-PAGE OF HAPTENISED MOUSE SKIN MICROSOMAL PROTEIN

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As an approach to resolve the haptenised proteins involved in the production of dermatoses 2, 4-dinitrochlorobenzene and 2, 4-dinitrofluorobenzene were chosen as model haptens with a well established and understood mechanism of protein preactivity. These haptens were applied to mouse skin *in Vivo*. Prior to sacrifice after 6 days, control and treated skin was homogenized and the microsomal proteins examined by dialysis, gel permeation and SDS-PAGE. Rather unexpectedly only one protein band in DNFB treated skin, appeared to have become labelled with an apparent molecular mass of 47,000 Da.

Key words: Hapten, Microsomal Proteins, SDS-PAGE.

Introduction

Allergic Contact Dermatitis (ACD) due to natural and synthetic products has been recognized for many years. The underlying immunological process involved in the production of contact sensitization has been elucidated but many details on the chemical aspects of the exact mechanism are still to be investigated. It has long been accepted that administration of contact sensitizers by intravenous, subcutaneous or intraperitoneal routes normally results in failure to induce sensitization reactions (Magnusson and Kligman 1983). However, epicutaneous or intradermal treatments usually induce sensitization reactions. This proves that a local interaction between the sensitizers and host proteins to produce altered proteins, which the body then recognises as foreign, is essential for the induction of contact sensitivity (Land steiner and Jacob 1963). It has been established that inducers of sensitivity are chemicals, which conjugate with proteins, while those which fail to conjugate lack sensitizing effects. Evidence in support of these findings emerged from sensitization experiments using hapten protein conjugates, hapten-amino acid conjugates and hapten-microsomal proteins prepared from hapten treated skin (Nishioka *et al* 1971). Despite these findings, so far little or no evidence has been reported in support of any specific cutaneous moiety that may be acting as a receptor for haptens. The present studies report conjugation of DNFB to a specific mouse skin microsomal protein wherein haptens conjugate to produce adducts during the induction elicitation of contact sensitivity.

Materials and Methods

General solvent and reagents were obtained from BDH Poole, England UK. Aldrich Chemicals Co. Milwaukee USA, and redistilled before use. 2, 4-dinitrochlorobenzene, 2, 4-Dinitrofluorobenzene were obtained from Aldrich Chemical Co. High molecular mass proteins calibration kit and Sephadex G-150 were obtained from Pharmacia Sweden. Electrophoretic chemicals acrylamide, N, N-methylenebisacrylamide, tetramethylenediamine (TEMED) 2, mercaptoethanol, Coomassie brilliant blue R-250 were obtained from Sigma Chemicals Co. St. Louis, MO, USA. For contact sensitization WSP male mice were used. Tissue homogenisation was achieved using a Ultrax-Turrax homogeniser (Janke and Kunkel, Werk UK) and Micro-ultrasonic cell disrupter (Kontes, Vineland, USA). Centrifugation of tissue homogenates was carried out using refrigerated centrifuge (MSE, UK). Electrophoresis of microsomal fractions was achieved using a slab gel unit (Shandon Scientific Co. UK).

Induction of contact sensitization. Male mice, 10 weeks old were divided into three 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 clipped. The concentration 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^{-1}), BNCB (2 mg 200 μl^{-1}) and DNFB (2 mg 200 μl^{-1}). A visual assessment of the skin reactions was made on day 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. Control and sensitized animals were sacrificed by cervical dislocations. The exposed skin in each group, which constituted about 20 percent of the total skin,

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was excised and immediately placed in ice cold 0.15 M potassium chloride solution.

Isolation of microsomes. Tissues homogenization and isolation of microsomes were carried out by the method of Bickers *et al* (1982).

Dialysis of microsomal fraction. A dialysis membrane was soaked in 0.1M phosphate buffer, pH 7.4 for 24 h. DNFB microsomal pellets 3 mg suspended in 1 ml 0.1M phosphate buffer pH 7.4 was transferred into the membrane, one end of which was knotted. The other end was also then sealed. The pouch thus obtained was placed in 200 ml glass beaker containing 150 ml 0.1M phosphate buffer, pH 7.4. The contents in the beaker were stirred by magnetic stirrer for 24 h at room temperature. Afterwards, the dialysed microsomal suspension was centrifuged at 100,000 rpm for 15 min. The supernatant and the pellet were separated.

Gel permeation of microsomal fraction. Sephadex G-150 particle size 40-120 μm weighing 6 g was hydrated with 100 ml 0.1μ phosphate buffer pH 7.4 and transferred into a glass column. DNFB microsomal pellet 5 mg, suspended in 1 ml 0.1M phosphate buffer, pH 7.4 and containing few crystals of bromophenol blue, was added on to the column. The column was eluted with 0.1 M phosphate buffer, pH 7.4 and 24 fractions, each containing 2 ml eluent were collected. These were centrifuged at 100,000 g for 15 min. The supernatants were discarded, and the pellets were retained for further processing.

SDS-Polyacrylamide gel electrophoresis of microsomal fraction. The method of Laemmli (1970) was used. A dis-continuous buffer system was employed. Stacking gel was polymerised on the top of the resolving gel. The microsomal pellets control, DNCB and DNFB were suspended in the sample buffer, and 20 μl of each suspension containing 90 μg microsomal proteins were applied. Marker proteins calibration kit vials were prepared according to the supplier's instructions. Content of each vial containing 5 marker proteins was dissolved in 100 μl sample buffer and heated at 60°C for 15 min. Before application, marker protein solutions were brought to room temperature. For use in SDS-PAGE, 20 μl marker proteins solution and microsomal protein samples were applied to separate wells. Before loading the wells, the upper buffer reservoir was filled with electrode buffer, and terminals were connected. The gel was run at 20 mA in the stacking gel and 35 mA in the resolving gel. Under these conditions, electrophoresis took about 7 h. When the tracking dye reached 0.5 cm from the bottom, the electrophoresis was stopped and the gel was taken out of the cassette. After recovery of the gel, the loaded wells were marked to identify the order of application of the samples. Similarly the position of the tracking dye was also marked, for Rf determinations. Protein bands on the gel were visualised by staining with Coomassie blue.

Results and Discussion

The results of homogenization and subcellular fractionation showed that DNFB microsomal fraction and unhomogenized skin residue were yellow whereas in case of DNCB, they were very faint. The observed difference in colour of microsomal fractions and skin residues suggests that DNFB accumulated at the test site in greater concentration compared to DNCB which had probably escaped with the result that less amount became available for binding. The same conclusion has been derived by Macher and Chase (1969), who found that more than 90% of DNCB escaped from the test site within one hour of the application to the skin.

The results of dialysis showed that the yellow product in DNFB microsomal fraction was not due to an impurity or free DNFB. Since DNFB is slightly soluble in aqueous solution, therefore its migration from one solution to another through the dialysis membrane was expected. However such migration was not observed. The results confirm conjugation of DNFB with microsomal proteins (Parker and Turk 1970).

Gel permeation of DNFB microsomal fraction yielded 24 fractions. Only fraction 8, 9 and 10 afforded yellow pellets and were combined.

For resolution of microsomal proteins on SDS-PAGE, different concentrations of polyacrylamide ranging from 5 to 15% were tried but better resolution of various protein bands was achieved with 10% concentration. The relative mobilities of various microsomal proteins are depicted in Fig1. As evident, control, DNCB and DNFB microsomal proteins exhibited distinct bands. These bands were numbered on the basis of their decreasing molecular mass band A (68,000 Da), B (57,000 Da), C (47,000 Da), D (36,000 Da). As evident from the Fig 1, DNFB eluted microsomal fraction (represen-ting subfractions 8, 9, 10) collected after gel permeation, showed the presence of a single protein band (47,000 Da).

Theoretically DNFB would be expected to bind covalently with many proteins. During *in Vivo* experiments on mouse skin, we observed that DNFB conjugated with only the microsomal fraction of the whole skin and furthermore with only one protein band in the SDS-PAGE experiments. The yellow colour of DNFB microsomal fraction allows justification of this conjugation. This is a rather unexpected observation and suggests the existence of a specific hapten receptor protein in the skin microsomes. The results indicate

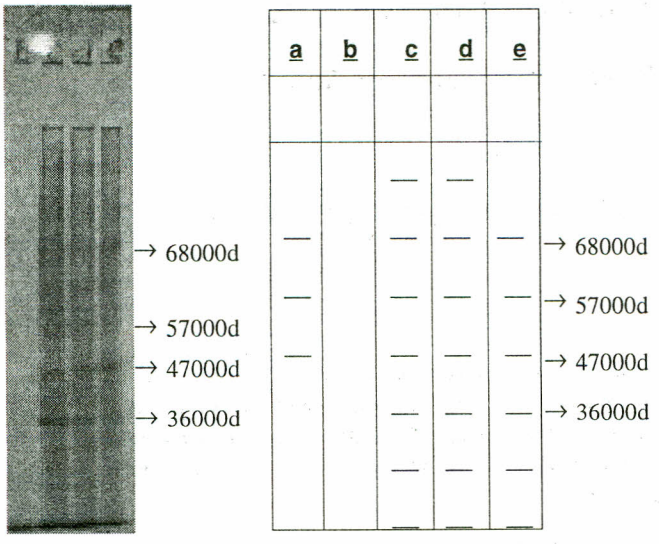

Fig 1. Electrophoretograms of mouse skin microsomal proteins. Slot No. (a) High molecular mass marker proteins, (b) DNFB Treated microsomal skin protein after gel permeation; (c) DNFB Treated microsomal skin proteins as such; (d) DNCB Treated microsomal skin proteins as such; (e) Control microsomal skin proteins as such.

that after penetration into the skin, DNFB conjugated with a specific endogenous protein moiety. A number of techniques have been developed over the past few decades to characterize carrier substances in allergic contact derma-titis by *in Vivo* experiments and some success has been reported in the induction of contact sensitivity with hapten-protein conjugates (Dupuis and Benezra 1982) hapten amino acid conjugates (Weck *et al* 1966) and hapten-microsomal protein conjugates (Nishioka *et al* 1971). However, carrier substances in allergic contact dermatitis remained ill defined. So far, little is known about the involvement of any specific protein moiety that might be acting for conjugation. In these investigations, evidence is presented which shows that after epicutaneous applications, DNFB conjugated with a specific protein moiety. The results also suggest that carrier substances involved in the production of dermatoses would be detectable and quantifiable in mouse skin. However, the limited ill defined data available in the literature make it difficult to draw general conclusion. Without characterization of receptor proteins, the observation would be a speculation. Therefore, further studies to characterise the adduct thus identifying the carrier substances would be of fundamental significance.

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