THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 57, No. 4

Printed in U.S.A.

THE DISTRIBUTION OF 2,4-DINITROPHENYL GROUPS IN GUINEA PIG SKIN FOLLOWING SURFACE APPLICATION OF 2,4-DINITROCHLOROBENZENE: AN IMMUNOFLUORESCENT STUDY*

SHOJIRO NAKAGAWA, M.D., HIROAKI UEKI, M.D. AND KIHEI TANIOKU, M.D.

ABSTRACT

The distribution of 2,4-dinitrophenyl (DNP-) groups in skin following surface application of 5 or 0.2 per cent solutions of 2,4-dinitrochlorobenzene (DNCB) in ethanol on the perioral or back skin of normal guinea pigs was investigated at varying times using an immunofluorescent method. It was demonstrated that DNCB penetrated through epidermis into dermis and then combined with the skin components within a few minutes after application. DNP-groups were clearly demonstrated in the cytoplasm of the epidermal cells and also detected in the histiocytic cells of the upper dermis.

In the specimens taken at 9 hours after application of a 5% solution of DNCB, necrosis of the epidermal cells was observed. The reparative process of epidermis first appeared in fifteen-hour lesions of the perioral skin or in two-day lesions of the back skin, and DNP-groups were not detected in the regenerating epidermis. In the three-day lesions of the perioral skin or the eleven-day lesions of the back skin, no fluorescent material was observed in either epidermis or dermis.

In the fifteen-hour and one-day lesions taken from the perioral skin to which was applied 0.2% solution of DNCB, the cells in the lower rete became poorly stained with the fluorescent antiserum. DNP-groups were detected only in the cornified outer part of epidermis in the two and three-day lesions and then disappeared in the five-day lesions. It is concluded that the disappearance of the greater part of DNP-groups conjugated with epidermal components may be ascribed to the shedding of the epidermis to exterior.

Several investigations in regard to the localization of allergens in the skin following surface application have been carried out to clarify the mechanism of allergic contact dermatitis. One of the important questions is what structures in the skin acquire the ability to induce contact hypersensitivity by combination with a simple chemical allergen? The answer to this question still remains unknown.

In attempts to study the localization of allergens, investigators have used various simple chemical allergens which are easily obtained in radioactive form, such as mercury (1), iodine (2) and 2,4-dinitrochlorobenzene (DNCB) (3). Eisen and Tabachnick observed the distribution of dinitrobenzene groups in guinea pig skin by means of radioautography and specific histological staining (3). Even with these methods the details of histological localization of the allergens have been inadequate.

In the present paper we have attempted to observe the cutaneous distribution of DNCB applied to the guinea pig skin surface. For this purpose, an immunofluorescent method using antiserum to 2,4-dinitrophenyl groups as a tracer of DNCB has been used.

Received February 17, 1971; accepted for publication May 27, 1971.

* From the Department of Dermatology, Okayama University Medical School, Shikatacho 2-5-1, 700 Okayama, Japan.

MATERIALS AND METHODS

Animals. Male albino guinea pigs, weighing 300 to 400 g, were used for observation of cutaneous distribution and 2.5 kg male rabbits were used for preparation of antisera to 2,4-dinitrophenyl (DNP-) groups.

Experimental materials. Normal guinea pigs were respectively given an application of 5% or 0.2% solution of 2,4-dinitrochlorobenzene (DNCB) in ethanol to the skin surface of the perioral region and the back by dropping on 0.1 ml of the solution. Two concentrations of DNCB at irritant or non irritant level were used. A full thickness biopsy from the application site was obtained under general anesthesia with ethyl ether,

at various intervals following application: 1 and 6 minutes, 3, 6, 9, 15 and 16 hours, and 1, 2, 3, 5, 9 and 11 days. Three to five biopsy specimens were examined at each interval.

Unfixed frozen sections prepared from a biopsy specimen of normal back skin of a guinea pig were immersed in a 5% solution of DNCB in ethanol for 24 hours, and washed sufficiently in ethanol to remove unreacted hapten. Thereafter, these sections were buffered in PBS-solution (0.01 M phosphate buffer saline, pH 7.2) for the immunofluorescent method.

Preparation of unfixed frozen sections. Some specimens were immediately frozen in an acetone dry ice chamber (−70° C), stored at −40° C, and cut at 5 μ on a cryostat at −25° C within 14 days. Each of the sections was placed on a glass slide, air dried and washed for 15 minutes in PBS-solution without fixation for the immunofluorescent method.

Preparation of paraffin sections. The paraffin embedding method by Hamashima, described previously (4), was used for the other specimens. The paraffin blocks were stored at 4° C. Sections were cut at 5 μ, placed on a glass slide, air-dried, and immersed in xylene to dissolve paraffin for one minute at 4° C. The xylene was then removed from the section in two successive changes of 100% anhydrous alcohol, and the sections were buffered in PBS-solution for the immunofluorescent method.

Preparation of DNP-protein conjugates. DNP-ovalbumin and DNP-guinea pig serum were prepared by minor modifications of the procedure of Parker, Kern and Eisen (5). Eighty-five mg ovalbumin or guinea pig serum, 28 mg 2,4-dinitrobenzene sulfonic acid sodium salt ($DNBSO_3$ Na) and 48 mg K_2CO_3 were dissolved in 10 ml water and shaken slowly overnight at room temperature. The solution was dialyzed for 4 days at 4° C against PBS solution to remove unreacted hapten and dinitrophenol. At the end of this procedure, a small amount of insoluble material was removed by centrifugation.

Preparation of antisera. Three rabbits were given five weekly injections of 8 mg DNP-ovalbumin. For the first two injections, the conjugate was emulsified with an equal volume of Freund's complete adjuvant (Difco) and given in the footpads. The other injections were given subcutaneously in the back without adjuvant. Three weeks after the last injection, sera were obtained and tested for precipitating antibody by the Ouchterlony method. One of the sera showed two precipitin arcs on the agar gel double diffusion to DNP-ovalbumin and an arc to DNP-guinea pig serum, but not to guinea pig serum (Fig. 1). There was no precipitation line between the antiserum and $DNBSO_3$ Na or 2,4-dinitrophenol. These findings show that the antiserum is specifically directed toward the hapten portion, that is DNP-groups, of the conjugates and does not react with uncoupled hapten. Titer of the antiserum was 1:32.

Immunofluorescent method. Antiserum was fractionated with one-third saturated ammonium

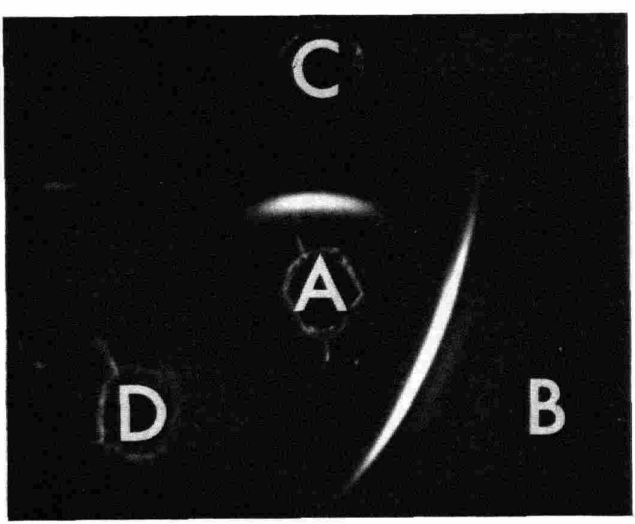

FIG. 1. Double diffusion in agar gel. (A), Rabbit anti-2,4-dinitrophenyl (DNP-) ovalbumin serum, ½; (B), DNP-ovalbumin, 2 mg/ml; (C), DNP-guinea pig serum, 2 mg/ml; and (D), guinea pig serum, 2 mg/ml. The antiserum shows two precipitin arcs to DNP-ovalbumin and an arc to DNP-guinea pig serum.

sulfate, labeled with fluorescein isothiocyanate, and purified by column chromatography (Sephadex G-25 and DEAE-cellulose) by the procedure described previously (4). The fluorescein to protein ratio of the labeled antiserum was estimated to be 2.0. This antiserum was used after absorption with rabbit liver acetone powders. The treated sections were mounted in polyvinyl alcohol:PBS solution:glycerin (2:8:5) (Elvanol) and submitted to fluorescent-microscopic examination using a fluorescence microscope with an ultraviolet exciting filter and yellow barrier filter.

Each section which had been investigated with the immunofluorescent method was stained again with hematoxylin and eosin for observation of histologic changes.

For controls, the blocking tests by unlabeled antiserum and specific antigen were carried out. No specific fluorescence was observed in the section treated with fluorescent antiserum to human IgG and in the sections from normal or croton oil painted guinea pig skin treated with the labeled antiserum to DNP groups. Furthermore, 5% DNCB was applied to the surface of frozen guinea pig skin, and the section obtained from the site was treated with the labeled antiserum to DNP-groups. The specific fluorescence was not found in the skin. The possibility that the penetration of DNCB might develop in the specimens while being stored, was excluded by this experiment.

RESULTS

Distribution of DNP-groups in the skin where a 5% solution of DNCB was applied. One minute after DNCB in ethanol had been applied to the skin surface in the perioral region of normal guinea pigs, the epidermis, except horny layer, was stained greenish yellow with fluorescent antiserum. Fluorescence was particularly brilliant on the granular and basal layers. The nuclei of the epidermal cells were not stained. Fluorescence was also found on the outer sheath of the hair follicles, arrector pili muscles and sebaceous glands. In the six-minute lesions, fluorescent materials were seen on the outer part of the horny layer, but the horny layer itself was not stained (Fig. 2). Fluorescence on the granular and basal layers was more obvious. Numerous feather-like or point-shaped materials were fluorescent in the upper dermis in the unfixed frozen sections. In the paraffin sections, the epidermis, except horny layer, was diffusely stained and fluorescence was also distributed in the areas corresponding to cells and collagen bundles in the upper dermis.

Three-hour specimens showed diffuse fluorescence in the epidermis, except the horny layer. The outlines of the cytoplasm of the epidermal cells were clearly recognizable with brilliant fluorescence (Fig. 3). There was no fluorescence in the dermis except follicles and cells.

In six-hour specimens, epidermal alterations were seen. The cells stained poorly. The atrophic epidermis became detached partially from the dermis. The horny layer was stained. The inflammatory cells, predominantly polymorphs, began to infiltrate around the dilated capillaries in the upper dermis and under the cleavages. In the unfixed frozen sections, fluorescence was also detected in the histiocytic cells of the upper dermis (Fig. 4). Polymorphs were not observed because of having been washed out in the processing.

The total epidermal layer with complete epidermal necrosis was separated from the dermis in the nine-hour lesions. The dermal infiltration

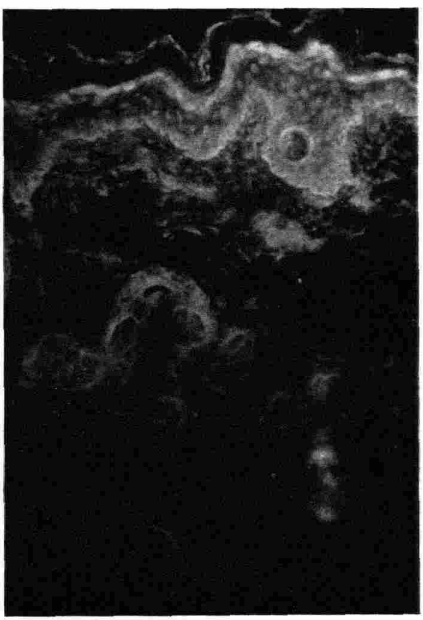

FIG. 2. Section obtained from the skin of perioral region in a normal guinea pig 6 minutes after surface application of 5% 2,4-dinitrochlorobenzene (DNCB) in ethanol, stained with fluorescent antiserum to DNP-groups. Fluorescence is observed in the epidermal layer, the outer sheath of hair follicles and sebaceous glands. Numerous feather-like and point-shaped materials with fluorescence accumulate in the subepidermal region of dermis (unfixed frozen section, × 120).

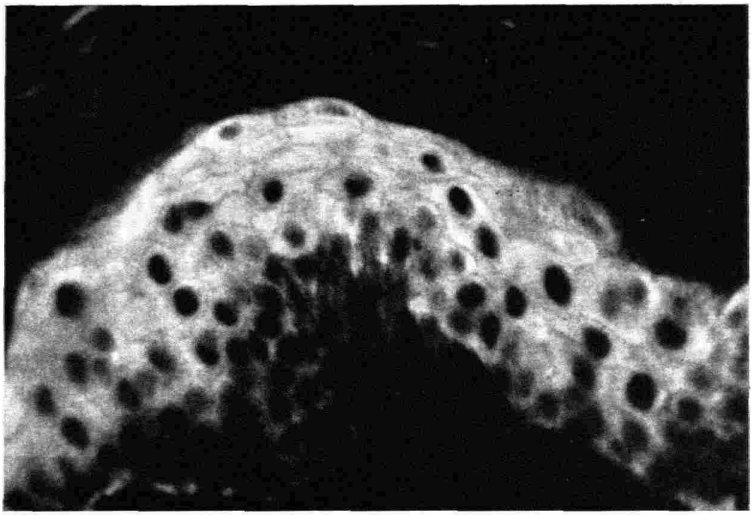

FIG. 3. Section of the perioral region 3 hours after application. Fluorescence is distributed diffusely in the epidermal layer, except the horny layer, and observed in the areas corresponding to the cytoplasm of epidermal cells (paraffin section, × 300).

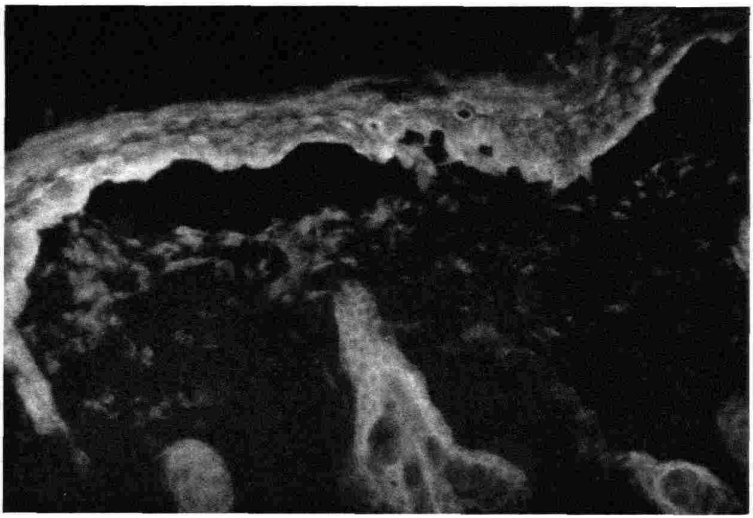

FIG. 4. Six-hour lesion obtained from the skin of perioral region. The epidermis is detached from the dermis. Fluorescence is detected in histiocytic cells of the upper dermis (unfixed frozen section, × 150).

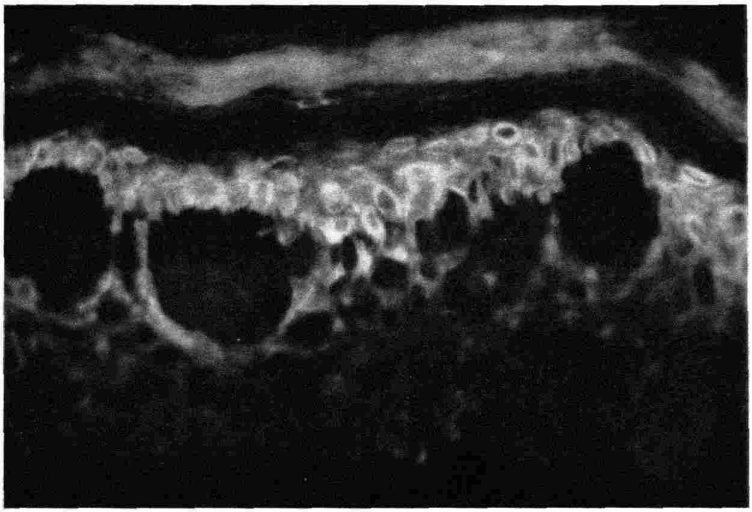

FIG. 5. Section of the perioral region 15 hours after application. Spongiotic areas are observed in the middle rete. The epidermal cells under the spongiotic areas are poorly stained (unfixed frozen section, × 300).

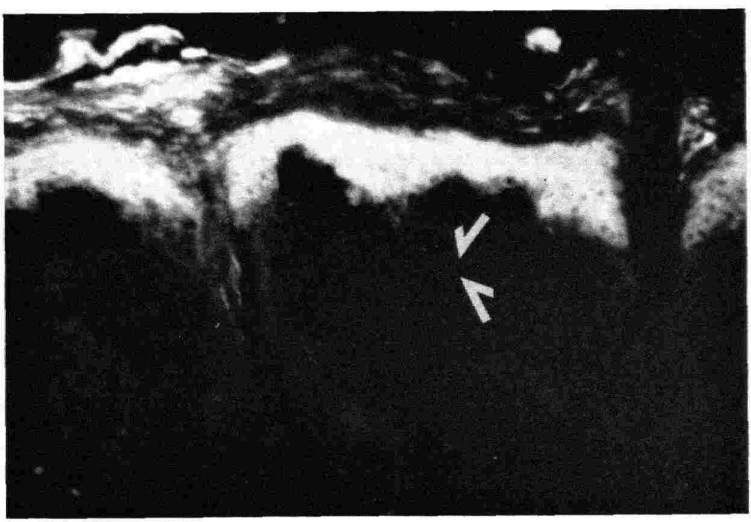

FIG. 6. Sixteen-hour lesion from the perioral region. In the lower rete, an area corresponding to the regenerated epidermal layer, fluorescence is not demonstrated. Arrows show the dermal-epidermal junction of the new epidermis (paraffin section, × 150).

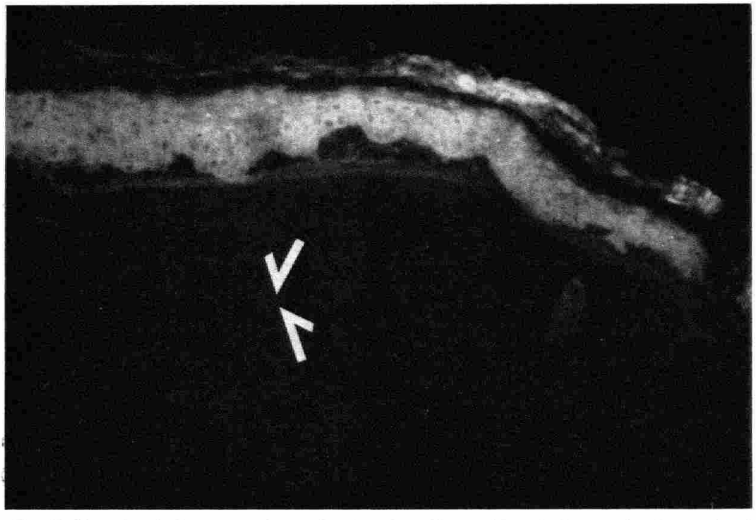

FIG. 7. Two-day lesion from the perioral region. The epidermis is completely repaired. Fluorescence is confined to the old epidermal layer. The outlines of fluorescent cytoplasm of epidermal cells are recognizable. Arrows show the dermo-epidermal junction (unfixed frozen section, × 150).

by inflammatory cells increased and the inflammatory elements migrated into the epidermis and the cleavages in the paraffin sections. The localization of the fluorescence in these cells could not be demonstrated with certainty.

In the fifteen-hour lesions, a reparative process became apparent in the epidermal lining of the upper part of the hair follicles. The cells in the lower rete took the stain poorly (Fig. 5). There was no fluorescence in the dermis except in the hair follicles. We noticed an unstained layer over the granular layer as shown in Fig. 5. In a sixteen-hour lesion, a regenerated epidermal layer, two to three cells thick, covered the entire dermal surface and was overlaid with the dead epidermis. Spongiotic areas were observed between the two epidermal layers. The new epidermal layer was not stained (Fig. 6).

In the specimens taken at one day after application, the new epidermal layer became four to six cells thick, and two-day lesions showed a completely regenerated epidermis. Fluorescence was detected only in the old epidermal layer (Fig. 7). In the three-day lesions, the old epidermal layer had sloughed off, and no fluorescent material was observed in either epidermis or dermis.

The histologic changes and the distribution of DNP groups in the specimens taken from the back skin receiving 5% solution of DNCB in ethanol were not fundamentally different from the above-mentioned observations except for the time course of the histologic changes. The regeneration of epidermis appeared later than that of the skin specimens taken from the perioral skin, first noted in the two-day lesions and a nearly completely regenerated epidermis was observed in the five-day lesions. The DNP groups had disappeared from the skin in the eleven-day lesions.

The findings described above are summarized schematically in Figure 9.

In the sections immersed *in vitro* in 5% solution of DNCB in ethanol for 24 hours, the epidermis was poorly stained with the fluorescent antiserum. The specific fluorescence was observed in some of the prickle cells. It was also detected partially in the topmost horny layer. In contrast, the collagen bundles were uniformly stained.

Distribution of DNP groups in the skin where a 0.2% solution of DNCB was applied. One and six minutes after application of DNCB to the perioral skin of normal guinea pigs, fluorescence

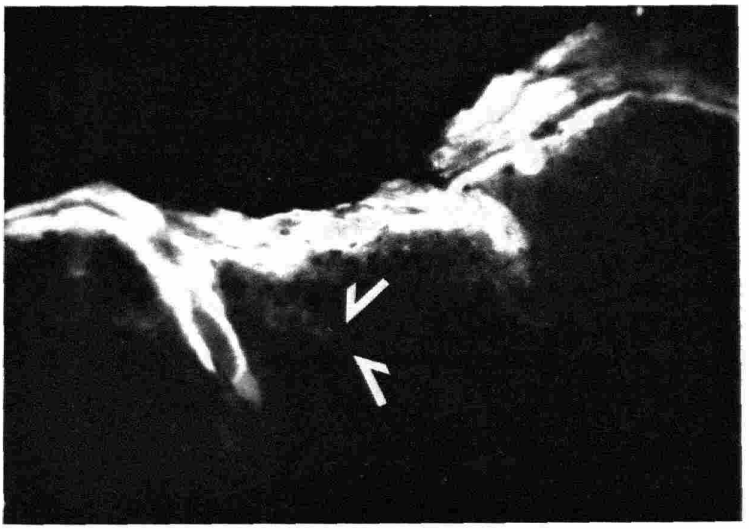

FIG. 8. Section from the perioral region 2 days after application of 0.2% DNCB in ethanol. Fluorescence is confined to the area corresponding to the horny and granular layers. Arrows show the dermo-epidermal junction (unfixed frozen section, × 150).

was observed in the epidermis except for the horny layer and appeared to be marked on the upper portion of the rete. In the specimens taken at three to nine hours after application, the epidermis, except the horny layer, was diffusely stained. The outlines of the cytoplasm of the epidermal cells were also recognizable with fluorescence. The cells in the lower rete were poorly stained in the fifteen-hour and one-day lesions. The fluorescence was confined to the areas corresponding to the granular and horny layers in the two and three-day lesions (Fig. 8). In the five-day lesions, we could not observe any fluorescence in the skin.

DISCUSSION

A technical point, different from a previous study with regard to the cutaneous distribution of DNP groups (3), should be pointed out. Histologic examination by immunofluorescense could detect only the DNP groups. However, there is no fundamental difference between our result and the previous observations with respect to cutaneous distribution of DNP groups.

The observation in the present study supports the finding that DNCB penetrates through the epidermis into the dermis and beyond and com-

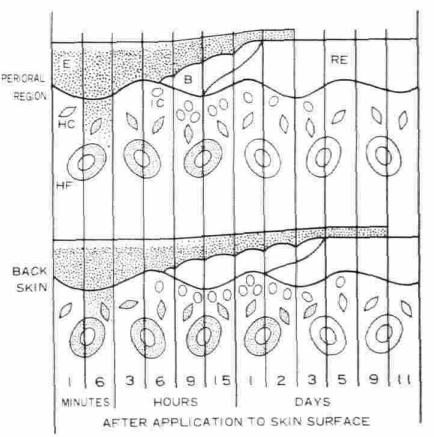

FIG. 9. Schematic summary of cutaneous distribution of DNP-groups at different times after surface application of 5% DNCB in ethanol to the skin of the perioral region or the back in normal guinea pigs. Dots represent DNP-groups, not extractable with ethanol and PBS-solution, and possessing an ability to react with the antiserum, localized by immunofluorescent method. (E), Epidermis; (HC), histiocytic cells; (HF), hair follicles; (IC), infiltrating cells; (B), bullae; and (RE), regenerated epidermis.

bines with skin components within a few minutes after application to the skin surface. As indicated by Feldman and Maibach (6), DNCB penetrates so extensively as to suggest that the guinea pig skin has little barrier properties to the compound. The combination of DNCB with skin components is also very rapid, comfirming the result of Eisen and Tabachnick (3). As shown in Figure 2, the fluorescent materials accumulate in the subepidermal region. It is reasonable to assume that the actual route of penetration of DNCB into dermis is transepidermal. It has been shown by several previous studies (3, 6, 7), directed to tracing the fate of DNCB or some derivatives of it following percutaneous application, that a large portion of the sensitizer escapes rapidly from the skin via regional lymphatics or veins and is distributed in non-cutaneous tissues or excreted in urine. Only about 5 per cent of the applied DNCB remained in the skin 24 hours after surface application to guinea pig skin in the work of Eisen and Tabachnick (3). It should be emphasized that only the portion of the applied sensitizer which remains in the skin is detectable in the histological analysis of the skin for localization.

The specific fluorescence was distributed diffusely in the epidermal layer except for the horny layer in the three-hour lesions, irrespective of the concentration used for application or the site of applied DNCB. It was clearly observed in the cytoplasm of epidermal cells. This finding suggests a marked affinity between the cytoplasmic components and DNCB. Most recently, Parker and Turk (8) reported that the DNP-microsomal fraction of epidermis obtained from the DNFB painted guinea pig skin was found to be statistically more antigenic than either the DNP mitochondrial fraction or the soluble conjugates in the cell sap in the induction of contact sensitivity. This finding suggests that the cytoplasmic components of epidermal cells may play a role in the development of contact sensitivity. It was reported by Klaschka (9), however, that in epicutaneous DNCB sensitization *in vivo* the hapten was likely to conjugate with the membranes of epithelial cells or with intercellular substances to form the antigen.

It has also been demonstrated that the area of skin on which DNCB has been applied can be removed 24 hours or longer after application without preventing the development of contact sensitivity (10, 11). Excision of the site before this time prevents the development of sensitization. Furthermore, Eisen and Tabachnick (3) pointed out that the allergic response was elicitable when bound dinitrobenzene groups were present in the vacinity of the epidermal-dermal junction. It is suggested that DNP groups detected in the skin, especially in the epidermis in the present study, may contribute to the induction of contact sensitivity.

As shown in Figure 4, the fluorescence could also be detected in histiocytic cells of upper dermis. It might be expected that DNP groups are taken into these cells by phagocytic mechanisms. If this interpretation is correct, the following possibility is suggested. The dermal histiocytic cells phagocytize the soluble epidermal components conjugated with DNCB or the cytoplasmic components which have been combined with DNCB in the epidermal cells. Macher and Chase (12) reported that only the portion of the sensitizer that remained in the skin between 12–24 hours and 3–4 days was antigenic and the immunological information for sensitization was picked up *in situ* ("peripheral sensitization"). It seems plausible that DNP groups detected in the dermal histiocytic cells participate in the development of contact sensitivity. It is not possible to rule out completely, however, that free DNCB diffusing from the skin surface would react as readily with the components of dermal cells as with those of epidermal cells.

Low reactivity of collagen fibers with DNCB *in vivo* was demonstrated, confirming the result of Eisen and Tabachnick (3). However, the study performed *in vitro* shows a considerable affinity between collagen fibers and DNCB. This discrepancy may be explained by the presence of some factor *in vivo*. Low reactivity of horn cells with DNCB was also demonstrated *in vivo* and *in vitro*. Fluorescence was not observed in the horny layer for up to 3–6 hours *in vivo*. It is assumed from these findings that the granular and prickle cells conjugated with DNCP eventually appear as cornified cells in the keratinization process.

The reparative process of epidermis correlated with the disappearance of fluorescence from the lower part of the epidermal layer in the specimens which had 5% DNCB applied. When the old necrotic epidermis sloughed off, fluorescence disappeared completely. It was observed that loss of DNP groups in the epidermal cells to which 0.2% DNCB was applied was also associ

ated with shedding of epidermal scales. These observations suggest that the disappearance of the greater part of DNP groups combined with epidermal components may be ascribed to the shedding of the epidermis, as pointed out by Eisen and Tabachnick (3).

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