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STUDY ON THE *IN VIVO* AND *IN VITRO* REACTIVITY OF GUINEA PIG SKIN WITH DINITROBENZENE COMPOUNDS

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Abstract

The distribution of DNP groups in guinea pig skin following surface application of DNCB in various solvents was investigated by immunofluorescent and immunohistochemical methods. There was no fundamental difference in the distribution by varying the solvents. DNP groups were detected in horny and Malpigian layers, but not in the so-called transition layer and collagen bundles. In vitro, the affiinity between DNCB and either epidermis except transition layer or collagen bundles was demonstrated. The significance of these findings is discussed.

INTRODUCTION

According to widely accepted conception concerning mechanism of contact sensitivity, the antigens are formed *in vivo* by conjugation of simple chemicals with skin proteins, especially epidermal proteins. The antigenic determinants involved in contact sensitivity include not only the hapten itself but also the autologous carrier proteins with which it has conjugated in the skin.[1,2,3]

In the previous study, histological analysis of skin for localization of 2, 4-dinitrochlorobenzene (DNCB) applied to the skin surface has been done, and it has been shown that DNCB penetrates easily into the skin and combines with cytoplasmic constituents of epidermal cells.[4] This finding adds considerable weight to the concept described above. Ethanol has been used as a solvent in this work, because contact sensitivity has been effectively induced by painting with DNCB in ethanol.[1,2] The use of the other solvents has been known to produce the sensitivity.

So the present work extends the observation using the same experimental method to DNCB in several other solvents. The method consists of an application of the compound to the guinea pig skin surface and a histological observation of its distribution in the skin by immunofluorescent and immunohistochemical methods using the antibody against 2,

4-dinitrophenyl (DNP) groups. The *in vitro* reactivity of DNCB with skin structures has been also examined.

MATERIALS AND METHODS

Experimental materials. Normal guinea pigs were given an application of 0.2 or 5 per cent solution of DNCB in various solvents or 0.3 per cent solution of 2, 4 dinitrobenzene sulfonic acid sodium salt ($DNBSO_3Na$) in PBS. Biopsy specimens were obtained from the skin sites 6 minutes and 3 or 24 hours after painting. Some specimens were taken from the skin to which 0.1 mg of DNCB in ethanol was injected intradermally. Unfixed frozen and paraffin sections were prepared from the specimens according to the previously described method.[4]

Unfixed frozen sections prepared from the perioral skin of guinea pig were immersed and shaken slowly at room temperature for 24 hours in 0.2 per cent solution of DNCB in the various solvent or in 0.3 per cent solution of $DNBSO_3Na$ in buffer at various pH values. Thereafter these sections were washed sufficiently in each solvent to remove unreacted compound and buffered in PBS for the immunofluorescent and immunohistochemical techniques.

Immunofluorescent and immunohistochemical methods. The fluorescein labelled antibody was prepared from rabbit antiserum to DNP groups as described previously.[4] A fluorescein to protein ratio of the labelled antibody was estimated to be 2.0. Investigations were carried out by direct immunofluorescent procedure.

Labelling with horseradish peroxidase (Sigma, Type II) was performed by the method of Nakane and Piece,[5] using p, p-dinitro m, m' dinitrophenyl sulfon as bifunctional reagent. The sections were treated with peroxidase-labelled antibody in the same way as conventional direct immunofluorescent procedure and stained for the enzyme by the method of Graham and Karnovsky.[6]

The blocking tests by unlabelled antiserum and specific antigens were carried out.

RESULTS

The distribution of DNP groups in the skin of guinea pigs following surface application of DNCB was investigated by immunofluorescent and immunochemical peroxidase procedures. The results are summarized in Table 1. The reaction product of the latter procedure appeared as dark brown precipitates. Fluorescence or reaction product was present in the epidermis except horny layer and localized in the cytoplasm

TABLE 1

The distribution of DNP groups in guinea pig skin following application of dinitrobenzene compounds in various solvents

Applied with	Horny layer	Transition layer	Malpigian layer	Collagen bundles
0.2 or 5% DNCB in ethanol				
6 mins after painting	−	−	+	−
3 hrs 〃 〃	−	−	+	−
24 hrs 〃 〃	+	−	+	−
0.2% DNCB in acetone				
24 hrs after painting	+	−	+	−
0.2% DNCB in olive oil				
24 hrs after painting	+	−	+～−*	−
0.2% DNCB in DMSO				
24 hrs after painting	+	−	+～−*	−
0.3% $DNBSO_3Na$ in PBS				
24 hrs after painting	+	−	−	−
0.1mg DNCB in ethanol				
24 hrs after intradermal injection	−	−	+	+

* The staining varies in the part of a section.

of the epidermal cells in the sections obtained 6 minutes and 3 hours after painting with DNCB in ethanol (Fig. 1 and 2). In 24-hour specimen, the horny layer became stained but reaction product was absent in a so-called transition layer as shown in Figure 3. The dermal collagen bundles were not stained in these sections. In the skin applied DNCB in olive oil and DMSO, staining was confined to the horny layer and the upper portion of Malpigian layer. Six-minute paraffin section showed diffuse staining through the dermis (Fig. 1), but almost all of it disappeared in the unfixed frozen section of the identical specimen. The collagen bundles and Malpigian layer were diffusely stained, but not the horny layer, when DNCB in ethanol was injected into dermis (Fig. 4).

The results relating the *in vitro* conjugation of DNCB or $DNBSO_3Na$ with skin are given in Table 2. Poor staining of the transition layer was also observed. The use of ethanol and alkaline buffers at pH values of 8 and 9 as solvent produced the conjugation of nuclei of prickle cells with DNCB and $DNBSO_3Na$. No staining was demonstrated in the skin immersed in $DNBSO_3Na$ at pH value of 5.

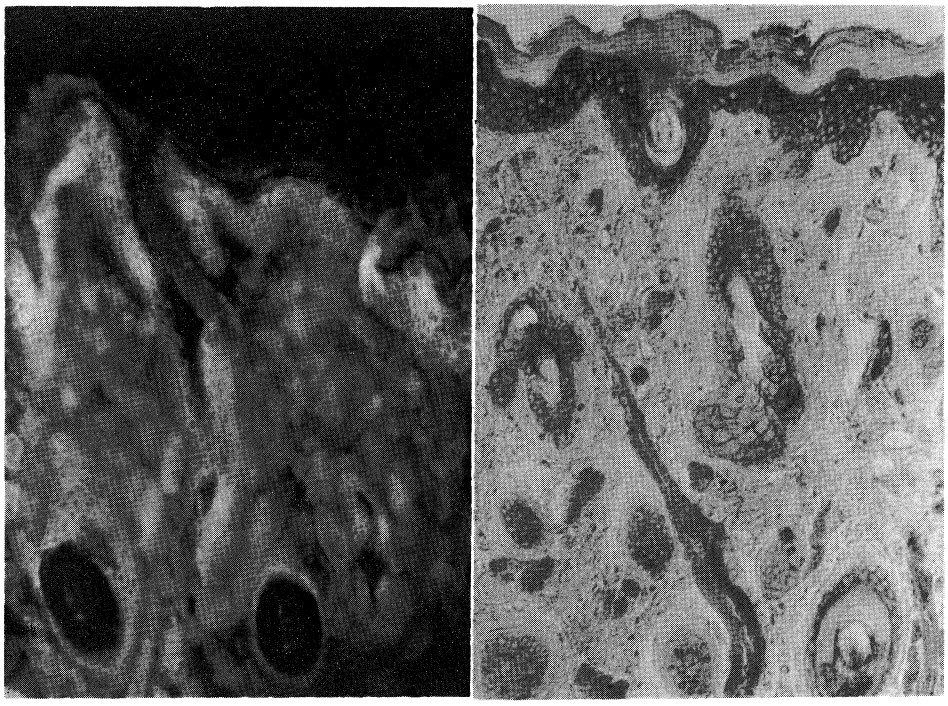

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Fig. 1. Section obtained from the skin of guinea pig 6 minutes after painting with 5% solution of DNCB in ethanol, stained with fluorescent antibody to DNP groups. Fluorescence is observed diffusely in epidermis except horny layer, and dermis (paraffin section, original magnification ×150).

Fig. 2. Section 3 hours after painting. The dark precipitates of reaction product of immunochemical peroxidase procedure are demonstrated in epidermis except horny layer and epidermal appendages, but not in collagen bundles (paraffin section, ×120).

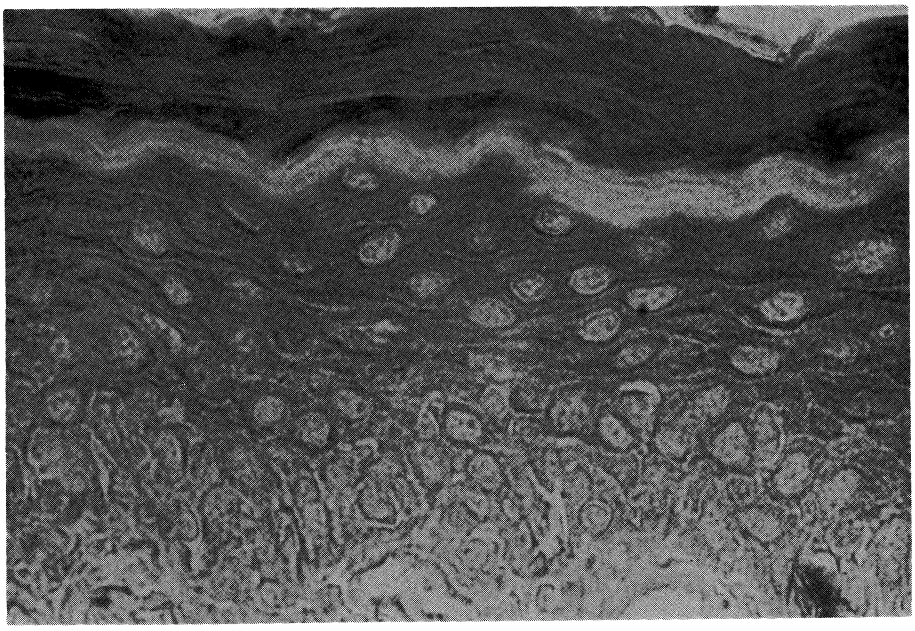

Fig. 3. Twenty-four hour lesion. We can see the reaction product in the horny layer and in the cytoplasms of prickle cells. Note unstained transition layer of the horny layer (unfixed frozen section, ×300).

TABLE 2

The distribution of DNP groups in sections of guinea pig skin immersed in dinitrobenzene compounds in various solvents for 24 hours

Immersed in	Horny layer	Transition layer	Malpigian layer	Collagen bundles
0.2 or 5% DNCB in ethanol	+~−*	−	+~−*	+~−*
0.2% DNCB in acetone	+~−*	−	+	+ −*
0.2% DNCB in olive oil	−	−	+	+
0.2% DNCB in DMSO	+	+~−*	+~−*	+~−*
0.3% $DNBSO_3Na$ in buffers				
pH 11	+	+~−*	+	+
pH 9	+	−	+	+
pH 8	+	−	+	+
pH 7	+	−	+	−
pH 6	+	−	+	−
pH 5	−	−	−	−

* The staining varies in the part of a section.

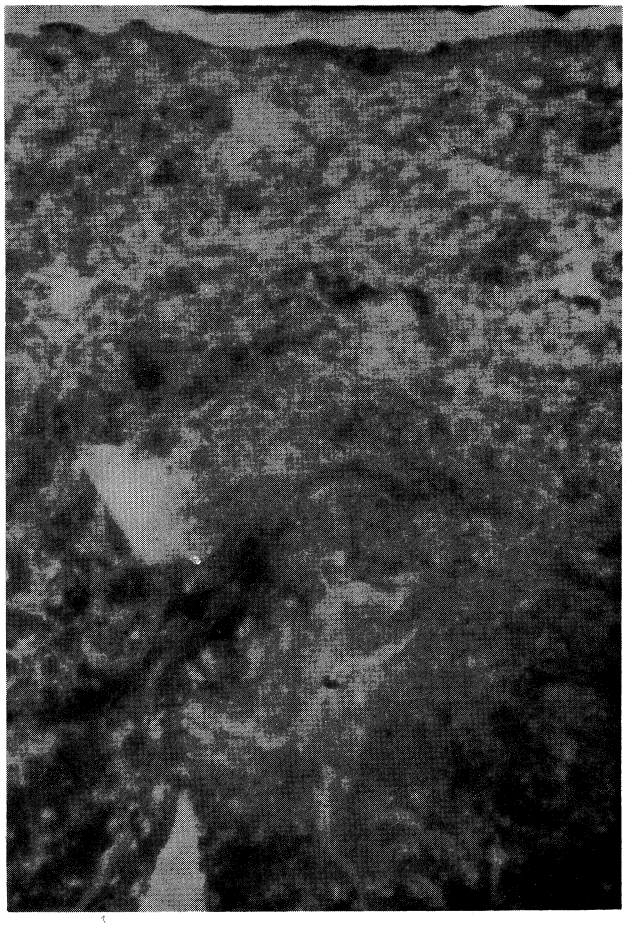

Fig. 4. Section obtained from the skin injected intradermally 0.1 mg of DNCB in ethanol 24 hours before. Diffuse fluorescent epidermis and numerous feather-like or point-shaped materials with fluorescence are observed (unfixed frozen section, ×120).

DISCUSSION

There were no fundamental differences in the *in vivo* distribution of DNP groups in guinea pig skin by examining DNCB in several solvents. DNP groups were uniformly demonstrated in the horny and Malpigian layers, but not in the so-called transition layer and collagen bundles. These findings show the possibility that the formation of antigens by conjugation of DNCB with epidermal proteins actually occurs in the skin painted with DNCB in all solvents.

The horny layer was not stained in the skin obtained 6 minutes and 3 hours after painting. The previous work[4] has shown that the localization of DNP groups in the horny layer occurs 6 hours or later. Furthermore, it has been demonstrated in the study that DNCB in ethanol combined with the cytoplasmic constituents of epidermal cells. The similar finding was obtained in the present work. It has been shown by several workers that DNCB couples *in vivo* to lysine ε NH_2 and free SH and SS groups of epidermal proteins.[7,8] *In vitro*, DNCB in several solvents and $DNBSO_3Na$ in buffers at various pH values also reacted with the epidermal structures except transition layer. Eisen et al. have reported that DNCB combines *in vitro* with lysine ε NH_2 and SH groups of hair and epidermis.[9] On the other hand, $DNBSO_3Na$ reacts with free SH groups and SS groups at physiological pH value, but reacts with lysine εNH_2 groups at high pH value of about 10. There was no reaction in the transition layer, either *in vivo* or *in vitro*. It is difficult to explain this observation. It is, however, of interest to call attention to the barrier effect of transition layer.[10]

When painted with DNCB in the several solvents to the skin surface, DNP groups were not detected in collagen bundles, confirming the results of several investigators.[4,10,11] On the other hand, the *in vitro* experiment showed a considerable affinity between collagen and DNCB. The intradermal injection of DNCB also made the conjugation with collagen bundles *in vivo*. It is reasonable to assume that the failure to detect DNP groups in the collagen bundles *in vivo* does not depend on the essential lack of reactivity between them, but on some factors *in vivo*. We are impressed by a low reactivity of collagen with $DNBSO_3Na$ at pH values of lower than 7.

In the 6-minute paraffin section, as shown in Figure 1, DNP groups were distributed diffusely in the dermis. But they were mostly washed out in the unfixed frozen section of the identical skin. This finding suggests that DNCB conjugates with soluble proteins in the skin. The presence of DNP-conjugates of serum proteins has been shown in the the skin painted with DNFB (2, 4-dinitrofluorobenzene).[12] Poor epidermal penetration of $DNBSO_3Na$ in PBS was demonstrated. It has been shown that with water as solvent without adding detergents $DNBSO_3Na$ is not effective for elicitating a positive contact reaction.[7]

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