

# *Acta Medica Okayama*

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*Volume 35, Issue 3*

1981

*Article 7*

JUNE 1981

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Demonstration of dnp groups on the draining lymph node cells of guinea pigs following skin painting with DNCB by peroxidase labelled antibody method.

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# Demonstration of dnp groups on the draining lymph node cells of guinea pigs following skin painting with DNCB by peroxidase labelled antibody method.\*

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## Abstract

The distribution of 2,4-dinitrophenyl (DNP) groups in the draining lymph nodes of guinea pigs 12 h after painting the skin with 2,4-dinitrochlorobenzene (DNCB) was examined by a peroxidase labelled antibody method using antibody against DNP groups. DNP groups were detected on cells that were found mainly in the subcapsular sinus of the lymph nodes. Electron microscopic examination showed DNP groups distributed on the surface of lymphocytes. The significance of these findings is discussed.

**KEYWORDS:** contact sensitivity, 2, 4-dinitrochlorobenzene, dinitrophenyl group, peroxidase.

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\*PMID: 6457511 [PubMed - indexed for MEDLINE]

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— BRIEF NOTE —

**DEMONSTRATION OF DNP GROUPS ON THE DRAINING  
LYMPH NODE CELLS OF GUINEA PIGS FOLLOWING  
SKIN PAINTING WITH DNCB BY PEROXIDASE  
LABELLED ANTIBODY METHOD**

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*Received February 16, 1981*

*Abstract.* The distribution of 2,4-dinitrophenyl (DNP) groups in the draining lymph nodes of guinea pigs 12h after painting the skin with 2,4-dinitrochlorobenzene (DNCB) was examined by a peroxidase labelled antibody method using antibody against DNP groups. DNP groups were detected on cells that were found mainly in the subcapsular sinus of the lymph nodes. Electron microscopic examination showed DNP groups distributed on the surface of lymphocytes. The significance of these findings is discussed.

*Key words :* contact sensitivity, 2,4-dinitrochlorobenzene, dinitrophenyl group, peroxidase.

The induction of contact sensitivity leading to immunogenic unresponsiveness has been extensively studied. Current studies using a variety of *in vitro* hapten modified cells (1-4) suggest that contact sensitizing agents may bind to lymphoid cells *in vivo* and, therefore, function in conjugation with certain membrane constituents as immunogenic and tolerogenic moieties. Our previous works using an immunofluorescent method (5-7) have established that the conjugation of a contact sensitizing agent with cell membrane actually occurs *in vivo* when 2,4-dinitrochlorobenzene (DNCB) is introduced into the body. In the experiment reported here, we continued our study of the distribution of 2,4-dinitrophenyl (DNP) groups on the surface of cells at the ultrastructural level.

*Materials and method. Animals.* Male Hartley strain guinea pigs weighing between 350-450g were used. *Production of anti-DNP antibody.* Hyperimmunized sera were obtained from rabbits that had been sensitized with DNP4-ovalbumin conjugate emulsified with Freund's complete adjuvant (Difco) as described previously(8). Anti-DNP antibody (anti-DNP) was specifically purified from these sera by an immunoabsorbent method according to Eisen *et al.* (9).

*Treatment of animals and preparation of specimens.* Guinea pigs were painted with a total of 0.5 ml of 5 % DNCB-ethanol solution on shaved areas of inguinal skin on both sides.

The inguinal lymph nodes were obtained from the animals 12 h after painting with DNCB. The nodes were cut into about 1 mm cubes and fixed in 4 % paraformaldehyde in PBS (0.01M phosphate buffer saline, pH 7.2) containing 8 % sucrose.

After washing in PBS with sucrose at 4°C overnight, blocks were frozen in PBS containing 20 % sucrose and 10 % glycerine. Cryostat sections of the frozen blocks (7µm) were mounted on glass slides coated with egg albumin, wash in PBS, then incubated with anti-DNP. The section were washed again in PBS, and incubated with horse-radish peroxidase labelled anti-rabbit IgG (Fab) (goat, M.B.L., USA). The section were washed sequentially in PBS, reacted with 3-3 diamino-benzidine peroxide and washed again. For electron microscopic examination, the sections were postfixed in 1 % osmium in sodium cacodylate buffer, dehydrated in graded ethanols and mounted in epon on glass slides as described by Yamada *et al.* (10).

Endogeneous peroxidase activity was previously blocked in 95 % methanol containing 0.3 % H<sub>2</sub>O<sub>2</sub> for light and electron microscopic sections. Blocking tests were performed by using peroxidase unlabelled anti-rabbit IgG. Control experiments were also performed by conventional techniques using anti-DNP antiserum absorbed with DNP-BSA and DNP-guinea pig albumin antigens.

**Results.** Twelve hours after a single painting with DNCB to the inguinal skin of guinea pigs, the draining lymph nodes showed marked enlargement compared to untreated animals. Histologically, this was due to edema in the subcapsular sinus, paracortical areas and medullary cords. Germinal centers were few and small. Reaction products indicative of DNP groups were distributed on the cell surface in the draining lymph nodes. Stained cells were seen mainly in the sub-

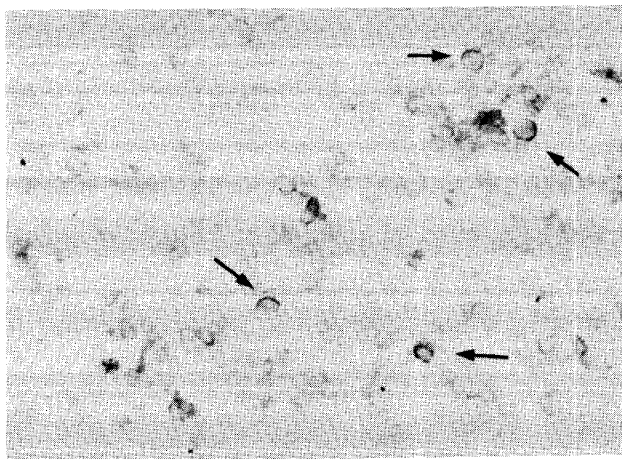


Fig. 1. A draining lymph node taken from a guinea pig 12 hours after skin painting with DNCB, labelled by an indirect technique of a peroxidase labelled antibody method. Frozen sections were treated with rabbit anti-DNP IgG, followed by peroxidase labelled anti-rabbit IgG antibody (goat). Reaction products are visible on the cell (—>) in the subcapsular sinus and the paracortical area of the lymph node. (X 400)

capsular sinus (Fig. 1). A few cells with reaction products were also observed in paracortical areas and medullary cords. Electron microscopic examination showed that DNP groups were distributed on the surface of the cells and that these cells had high nucleus-to-cytoplasm ratios (Fig. 2). The cytoplasm contained few cytoplasmic organelles. Rough endoplasmic reticulum, lysosomes and Golgi apparatus were not found in the cells. The cell population of stained cells was identified as lymphocytes morphologically. In control sections, reaction products were not seen on the cell surface in light microscopic sections of draining lymph nodes (Fig. 3) and, with electron microscopy, specific labelling of the cytoplasmic membrane of the cell was conspicuously absent.

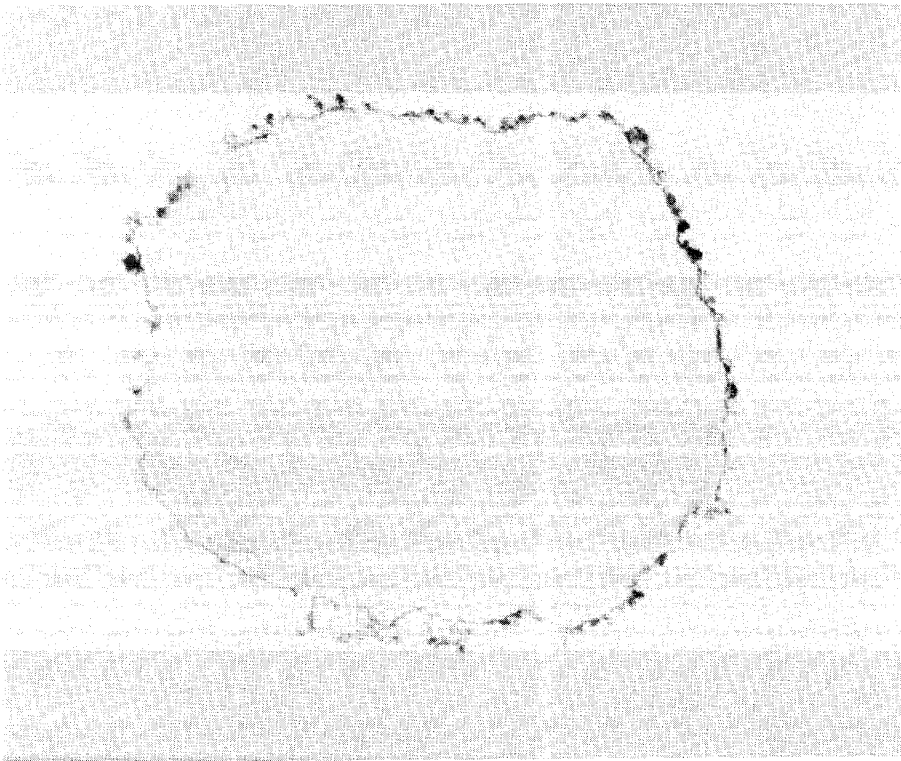


Fig. 2. An electron micrograph of a stained cell. Reaction products are seen on the surface of the cell. This DNP-positive cell is considered to be a lymphocyte by morphological criteria. (X 14,000)

*Discussion.* In our previous studies using this immunofluorescent method (6,7), DNP groups were shown to be distributed on cells of the peripheral lymphoid system of guinea pigs after DNCB had been painted on the skin. The majority of such cells (DNP cells) were found in the peripheral blood, spleen and thoracic duct 1-6 h after painting, but DNP cells reached a maximum in lymph

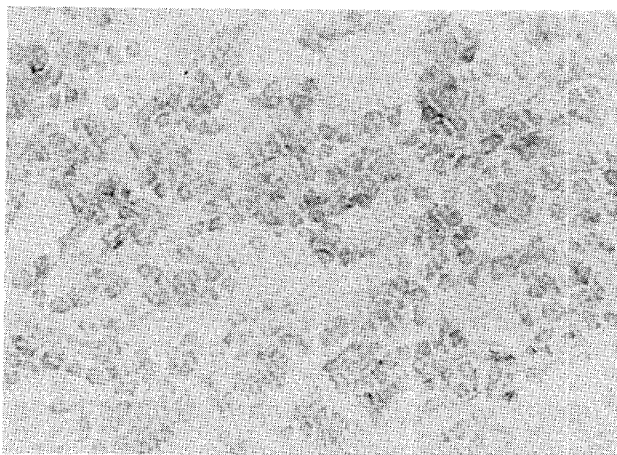


Fig. 3. A control section blocked by using peroxidase unlabelled anti-rabbit IgG. Reaction products are not seen on the cell surface of draining lymph nodes. (X 400)

nodes draining the site of DNCB application 12 h after painting. DNP cells were found mainly in the subcapsular sinus of the draining lymph nodes cells (11). Our study (6) indicated that, *in vivo*, DNCB reacts directly with cell membrane in the peripheral lymphoid system of guinea pigs following epicutaneous application of the agent. In addition, DNCB in its free unreactive form remained in the lymphoid system at least 12 h post-painting (6). However, the distribution of DNP groups on the cells is not clear by immunofluorescent method.

To investigate the distribution of DNP groups of cells in the draining lymph nodes more closely, we used peroxidase coupled anti-body as a visual marker so that the localization of DNP groups on the surface of the cells could be clearly followed by electron microscopy. This peroxidase staining showed that DNP groups were distributed on the surface of the cells. The majority of the DNP cells were lymphocytes. Other DNP positive cells were macrophages.

An important question raised by a study of this type, the kind of cell membrane protein to which the haptens bind *in vivo*. Forman *et al.* (12) demonstrated that trinitrophenyl groups directly couple to H-2 antigens on the cell surface of mouse spleen cells when treated *in vitro* with trinitrobenzen sulfonate. Other proteins including immunoglobulins are also derivatized with the hapten. Analysis of the hapten-lymphoid cell complex formed *in vivo* has not been done but, since previous data shows the existence of free unreactive hapten in the lymphoid system of hapten-painted animals (6), it is reasonable to assume that similar conjugation also occurs *in vivo* in the lymphoid system of animals painted with contact sensitizers.

*Acknowledgment.* We wish to express our thanks to prof. Nozomi Nohara for reviewing this manuscript.

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