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Ultrastructural Localization of DNP Groups on Draining Lymph Node Cells of Guinea Pigs Following Skin Painting with DNCB : II. Immunoferritin Electron Microscopic Study

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ABSTRACT. The distribution of DNP groups on the cells taken from the draining lymph nodes of guinea pigs 12 hours after painting the skin with DNCB was examined by transmission immunoelectron microscopy using the antibody against DNP groups and ferritin as visual marker. The study showed that DNP groups were distributed on the surface of lymphocytes, both T and B cells, and macrophages. It is suggested that DNCB penetrates through skin into lymphatics carrying lymph from the painted site and combines with the membrane of lymphocytes and macrophages in the peripheral lymphoid system and that the resulting modified cells act as immunogens or tolerogens in contact sensitivity.

Key words : DNCB — contact sensitivity — antigen distribution — immunoferritin electron microscopy

Contact sensitization is easily induced in animals by simply painting the skin with contact sensitizing agents. Suppressive mechanisms can be also introduced by contact painting. It has been shown that haptens coupled to autologous or syngeneic lymphoid cells *in vitro* are not only effective immunogens but also powerful tolerogens.[1-6] These current investigations using *in vitro* haptens modified lymphoid cells indicate that contact sensitizing agents bind to autologous lymphoid cells *in vivo* after introduced into body and, therefore, function in cojugation with certain membrane constituents as immunogenic and tolerogenic moieties. However, whether such conjugation of haptens with cell membrane actually occur *in vivo* remains to be established.

Our previous studies by immunofluorescent and scanning immunoelectron microscopic technics using antibody against 2,4-dinitrophenyl (DNP) groups have demonstrated that DNP groups are distributed on the surface of the cells in the draining lymph nodes, thoracic duct, spleen and peripheral blood when guinea pigs are painted the skin with 2,4-dinitrochlorobenzene (DNCB).[7-8] However, the cell population of the DNP distributed cells (DNP cell) is not clear by these methods. The purpose of the experiment in this report is to identify the cell population of DNP cells by immunoferritin electron microscopy.

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MATERIALS AND METHODS

Animals : Male Hartley strain guinea pigs weighing between 350–450 g were used.

Production of anti-DNP antibody : The hyperimmunized sera were obtained from the rabbits which had been sensitized with DNP_4–ovalbumin conjugate emulsified with Freund's complete adjuvant (Difco) as described previously.[10] Anti-DNP antibody (anti-DNP) was specifically purified from the sera by the immunoadsorbent method according to Eisen et al.[11] The prepared anti-DNP was characterized by gel diffusion and immunoelectrophoresis and was determined to be DNP specific IgG fraction.

Preparation of anti-DNP-ferritin conjugate (F-anti-DNP) : Horse spleen ferritin (F, twice crystallized, Nutritional Biological Co.) was further four times purified by cadmium sulfate recrystallization and ultracentrifugation. Conjugation of ferritin to anti-DNP and its purification were performed as follows.[12] Diluted GA was added to 400 mg ferritin in phosphate buffer 0. 1M, pH 7.0 to give a final concentration of 0.1% in a final volume of 5.0 ml and incubated for 2 hours at 37°C. The ferritin pretreated with GA (FGA) was filtered through a Sephadex G-25 column (50 cm × 2.5 cm) to remove unreacted GA. Anti-DNP was added to the affluent FGA in a 1 to 4 ratio in weight (final concentration of FGA; 280 mg/ml, anti-DNP; 70 mg/ml). The mixture was allowed to stand for 12 hours at 37°C without stirring. At the end of conjugation lysine was added to stop further cross linkage. Free anti-DNP and free ferritin were separated from the crude conjugate by preparative pevion electrophoresis.[13] This material was then loaded onto a sepharose 6B (70 cm × 5 cm) column equilibrated in 0.05 M tris/HCl buffer, pH 7.3.

Treatment of guinea pigs and preparation of specimens : Guinea pigs were painted with total 0.4 ml of 5% DNCB-ethanol solution on the shaved areas of both sides of inguinal skin. The inguinal lymph nodes were obtained from the animals at 12 hours after painting a large amount of Eagle's minimal essential medium (MEM). The cells were washed three times in MEM.

Rosetting procedures : In order to examine the lymphocytes subpopulation of the lymph node cells, the conventional E and EAC rosettes methods were carried out before the treatment of the cells with F-anti-DNP. For E rosette formation, washed packed rabbit red blood cells (E, 1×10^8) were added to 1 ml of lymph node cell suspension (1×10^6) containing 25% fetal calf serum, mixed thoroughly and then incubated overnight at 4°C. For EAC rosetting, sheep red blood cells treated with erythrocyte antibody (rabbit) and mouse complement (EAC, 1×10^8) were added to 1 ml of the lymph node cell suspension (1×10^6). The suspension was mixed and incubated at 37°C for 30 minutes.

Treatment of the lymph node cells with F-anti-DNP and preparation of samples for transmission electron microscopy : The lymph node cells were labelled with the F-anti-DNP (1 mg/ml) at 37°C for 30 minutes. After washing with phosphate buffer saline, 0.01 M, pH 7.2 three times, cell pellets were fixed in 2.5% GA in phosphate buffer (0.1 M, pH 7.4) and then 2% osmium tetraoxide in phosphate buffer for 1 hour at 4°C, respectively, dehydrated in graded ethanols, and embedded in Epon 812. Thin sections were stained with methanolic uranyl acetate and lead citrate, and examined under a Hitachi H-500 electron

microscope.

Control experiments : Normal lymph node cells and the draining lymph node cells from guinea pigs painted with oxazolone were treated with F-anti-DNP. The experiments were also controlled by conventional blocking techniques using

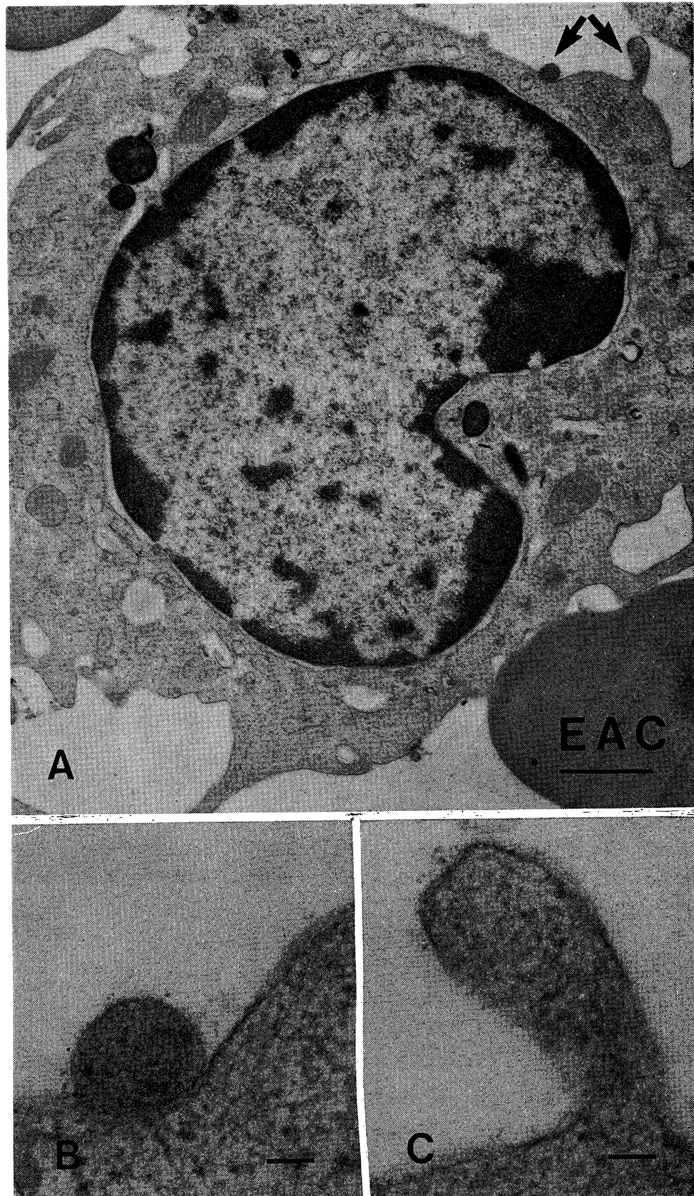

Fig. 1. Transmission electron micrographs of a lymphocyte in the draining lymph node taken from the guinea pig 12 hours after skin painting with DNCB, incubated with EAC and then ferritin-anti-DNP conjugate. *A*, A lymphocyte shows EAC rosette formation. Bar=1 μm. *B* and *C*, higher magnifications of the parts of the lymphocytes (arrows in panel A), Bars=0.1 μm. Villous projections show ferritin particles on their surface.

antibody and antigens.

RESULTS

The distribution of DNP groups on the cells taken from the draining lymph nodes of guinea pigs 12 hours after painting the skin with DNCB was

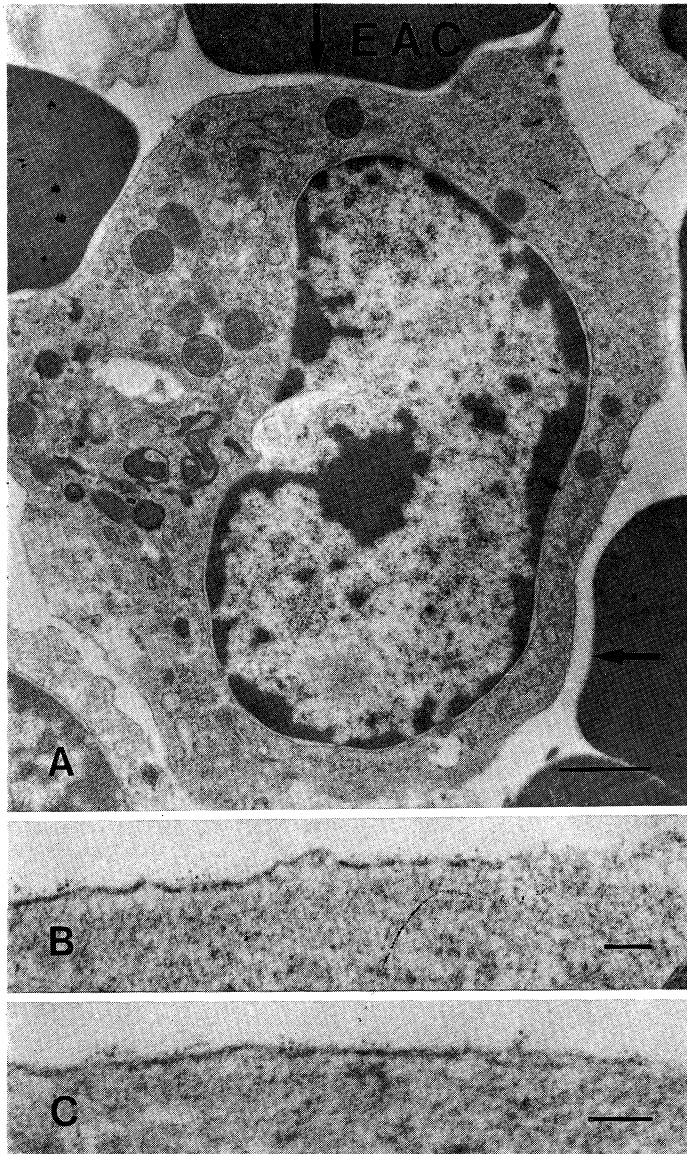

Fig. 2. A macrophage in the draining lymph node taken from the animal 12 hours after painting with DNCB. *A*, A macrophage forms rosette with EAC. Bar=1 μm. *B* and *C*, higher magnifications of the parts of the cells (arrows in panel A). Bars=0.1 μm. Ferritin is distributed over the cell surface.

examined by the direct method of the immunoelectron microscopy using F-anti-DNP. To determine the subpopulation of lymphocytes, E and EAC rosettes methods were carried out. F-anti-DNP were detectable on the surface of the cells which formed rosettes with either E or EAC. A part of the cells found with EAC were identified to be lymphocytes by the transmission electron microscopy and such cells (B cells) showed ferritin particles on their surface (Fig. 1). F-anti-DNP were also found on the surface of macrophages (Fig. 2).

DISCUSSION

In our previous studies using immunofluorescent and scanning immunoelectron microscopic methods, DNP groups were shown to be distributed on the surface of cells in peripheral lymphoid system of guinea pigs when DNCB was painted the skin.[7-9] The majority of DNP cells were found in the lymph nodes draining the site of DNCB application, thoracic duct, peripheral blood and spleen.[7-9] Using the immunoferritin electron microscopic method, it was confirmed in the present work that DNP cells were lymphocytes, both T and B cells, and macrophages. Forman et al.[14] have demonstrated that trinitrophenyl groups directly couples to H-2 antigens on the cell surface of mouse spleen cells when treated *in vitro* with trinitrobenzene sulfonate and that other proteins including immunoglobulins are also derivated with the hapten. Analysis of the hapten-lymphoid cell complexes formed *in vivo* has not been done but it is reasonable to assume from the previous data[8] that the similar conjugation also occurs *in vivo* in the lymphoid system of animals painted with contact sensitizers.

Contact sensitization and its suppression are achieved by injection of lymphoid cells conjugated *in vitro* with sensitizers.[1-6] Cells taken from draining lymph nodes of animals painted with contact sensitizers can induce contact sensitivity when injected into normal recipients.[15-19] Evidence shows injected cells immunize by transfer of antigen rather than transfer of potential effector cells.[15] This induction occurs when there is genetic matches at the major histocompatibility complex MHC between the donor and recipient animals.[19] The immunogenic activity in the draining lymph node cells was associated with T cells and macrophages if oxazolone was used as sensitizer.[16] In contrast, the experiment using fluorescein isothiocyanate as sensitizer immunogenecity was not associated with T cells.[19] Further studies have to be done in this experimental area.

It is suggested that contact sensitizing agents bind with various kinds of proteins including the products of MHC on the surface of the cells in the peripheral lymphoid system when introduced percutaneously, and that the complexes play important role as immunogens and tolerogens in contact sensitivity.

REFERENCES

1) Baumgarten, A. and Geczy, A.F. : Induction of delayed hypersensitivity by dinitrophenylated lymphocytes. Immunology **19** : 205-217, 1970
2) Polak, L. and Macher, E. : In vitro sensitization to dinitrochlorobenzene in guinea pigs. Nature **252** : 748-749, 1973
3) Nakagawa, S., Aoshima, T., Mimura, S., Gotoh, M. and Ueki, H. : The induction of contact sensitivity to DNCB with *in vitro* dinitrophenylated lymph node cells. Dermatologica **161** : 8-11, 1980

4) Miller, S.D. and Claman, H.N. : The induction of hapten-modified lymphoid cells. I. Characteristics of tolerance induction. J. Immunol. **117** : 1519–1526, 1976
5) Miller, S.D., Sy, M.S. and Claman, H.N. : Genetic restrictions for the induction of suppressor T cells by hapten-modified lymphoid cells in tolerance to l-fluoro-2, 4-dinitrobenzene contact sensitivity. Role of the H-2 region of the major histocompatibility complex. J. Exp. Med. **147** : 788–799, 1978
6) Miller, S.D., Conlon P.J., Sy M.S., Moorhead J.W., Colon, S., Grey, H.M. and Claman, H.N. : Nature of haptenmodified determinants involved in induction of T cell tolerance and suppressor T cells to DNFB contact sensitivity. J. Immunol. **124** : 1187–1193, 1980
7) Nakagawa, S., Amos, H.E., Gotoh, M. and Tanioku, K. : The distribution of 2, 4-dinitrophenyl groups in lymphoid tissue of guinea-pigs following skin painting with 2, 4-dinitrochlorobenzene. Immunology **36** : 851–857, 1979
8) Aoshima, T., Nakagawa, S., Gotoh, M. and Tanioku, K. : Studies on the dinitrophenylated lymphocytes in guinea pig painted with DNCB. J. Dermatol. **4** : 251–254, 1977
9) Nakaye, M., Nakagawa, S., Ueki, H., Kumon, H. and Ohmori, H. : Ultrastructural localization of 2, 4-dinitrophenyl groups on draining lymph node cells of guinea pigs following skin painting with 2, 4-dinitrochlorobenzene. J. Dermatol. **10** : 251–257, 1983
10) Nakagawa S., Ueki, H. and Tanioku, K. : The distribution of 2, 4-dinitrophenyl groups in guinea-pig skin following surface application of 2, 4-dinitrochlorobenzene. An immunofluorescent study. J. Invest. Dermatol. **57** : 269–277, 1971
11) Eisen, H.N., Grey, W.R., Russell-Little, J. and Sims, E.S. : In Methods in Immunology and Immunochemistry. Vol. 1, (Ed. by C. A. Williams and M. W. Chase), p. 355. 1967
12) Otto, H., Takamiya, H. and Vogt, A. : A two-stage method for crosslinking antibody globulin to ferritin by glutaraldehye. Comparison between the one-stage and two-stage method. J. Immunol. Methods **3** : 137–145, 1973
13) Batsford, S.R., Takamiya, H. and Vogt, A. : The isolation of an optimal small size immunoferritin conjugate fraction. J. Immunol. Methods **30** : 289–293, 1979
14) Forman, J., Vitetta, E.S., Hart, D.A. and Klein, J. : Relationship between trinitrophenyl and H-2 antigens on trinitrophenyl modified spleen cells. I. H-2 antigens on cells treated with trinitrobenzene sulfonic acid are derivated. J. Immunol. **118** : 797–802, 1977
15) Asherson, G.L. and Mayhew B. : Induction of cell-mediated immunity in the mouse : Circumstantial evidence for highly immunogenic antigen in the regional lymph nodes following skin painting with contact sensitizing agents. Israel. J. Med. Sci. **12** : 454–466, 1976
16) Asherson, G.L., Zembala, M. and Mayhew, B. : Analysis of the induction phase of contact sensitivity by footpad transfer of regional lymph node cells. Macrophages and radioresistant T lymphocytes induce immunity. Immunology **32** : 81–88, 1977
17) Thomas, W.R., Asherson G.L. and Perera, M.A.C.C. : Induction of contact sensitivity. Selective induction of delayed hypersensitivity by the injection of cells from draining nodes into the footpads of normal recipients. Immunology **34** : 725–731, 1978
18) Thomas, W.R., Edwards, A.J., Watkins, M.C. and Asherson, G.L. : Distribution of immunogenic cells after painting with the contact sensitizers fluorescein isothiocyanate and oxazolone. Different sensitizers from immunogenic complexes with different cell populations. Immunology **39** : 21–27, 1980
19) Asherson, G.L., Mayhew, B. and Perera, M.A.C.C. : The production of contact sensitivity by the injection into the footpads of recipients of the lymph node cells from mice 1 day after painting the skin with contact sensitizing agent : Requirement for matching at the major histocompatibility complex between donor and recipient mice. Immunology **37** : 241–245, 1979