Kawasaki Med. J. Vol. 5: No. 2, 103-113, 1979

DEMONSTRATION OF DNP GROUPS ON DRAINING LYMPH NODE CELLS IN GUINEA PIGS FOLLOWING SKIN PAINTING WITH DNCB BY SCANNING IMMUNOELECTRON MICROSCOPIC METHOD

Masako NAKAYÆ, Shojiro NAKAGAWA, Shigeru MIMURA, Hiroaki UEKI*, Hiromi KUMON and Hiroyuki OHMORI**

**Department of Dermatology, Kawasaki Medical School, Kurashiki 701-01 and*
***Department of Urology, Okayama University Medical School, Okayama 700, Japan.*

Accepted for Publication on November 26, 1979

Abstract

The distribution of DNP groups on the cells taken from the draining lymph nodes of guinea pigs 12 hours after painting skin with DNCB was examined by the indirect method of scanning immunoelectron microscopy using bacteriophage T4 labelled with antibody as visual marker. Fairly precise localization of DNP groups on the surface of the cells could be followed by the method. It was indicated that DNP groups were distributed in various amounts and in various patterns (diffuse, patchy or sparse) on the surface of the cells in the draining lymph nodes of animals when painted with DNCB. The incidence of the cells detected DNP groups on their surface was approximately 58%. The frequency of the cells with DNP groups was markedly reduced in the animals treated with DNBSO$_3$Na as compared to animals not receiving the tolerogen. The significance of these findings is discussed.

INTRODUCTION

The induction of contact sensitivity and production of immunogenic unresponsiveness in contact sensitivity systems have been extensively studied. The current studies using a variety of *in vitro* hapten modified cells[1-5] suggest that contact sensitizing agents may bind to autologous and, therefore, function in conjugation with certain membrane constituents as immunogenic and tolerogenic moieties. However, whether the conjugation of contact sensitizing agents with cell membrane actually occur *in vivo* when the allergens are introduced into body remains to be established.

中江昌子，中川昌次郎，三村　茂，植木宏明，公文裕巳，大森弘之

We have investigated the cellular localization of 2, 4-dinitrophenyl (DNP) groups in the peripheral lymphoid system of guinea pigs following percutaneous administration of dinitrobenzene compounds such as 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrobenzene sulfonate by immunofluorescent method using antibody against DNP groups[6-8]. Previous work has established that DNP groups are distributed mainly on lymphocytes in draining lymph node, spleen and peripheral blood when guinea pigs are painted the skin with DNCB[7]. The study also indicates that DNCB combines directly *in vivo* with membrane components of the cells when painted the skin[6].

The object of the experiments reported in this paper was to observe the distribution of DNP groups on the lymph node cells more closely by the scanning immunoelectron microscopic method using bacteriophage T4 labelled with antibody as visual markers. It was found that DNP groups were distributed in various patterns on the surface of lymph node cells and that the distribution of DNP groups on the cells varied according to whether treating the animals with cyclophosphamide or tolerogen painting the skin with DNCB.

MATERIALS AND METHODS

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

Production and characterization of anti-DNP antibody : The hyper-immunized sera were obtained from rabbits which had been sensitized with 2,4-dinitrophenyl $(DNP)_4$ ovalbumin emulsified with Freund's complete adjuvant (Difco, FCA) as described previously[9]. Anti-DNP antibody (anti-DNP) was specifically purified from the sera by the immunoadsorbent method according to Eisen et al[10]. The prepared anti-DNP was characterized by gel diffusion against DNP-protein conjugates and by immunoelectrophoresis with goat anti-rabbit whole serum and goat anti-rabbit IgG and was shown to be IgG fraction.

Preparation of antibody-marker conjugate : Goat anti-rabbit IgG sera were prepared by conventional method using FCA. IgG fraction was separated from the immunized pool sera by precipitated at 35% ammonium sulfate and chromatographed on DEAE cellulose. The pure IgG fraction was concentrated by vacum dialysis against phosphate buffer to a protein concentration of 35 mg/ml and stored at $-20°C$.

Bacteriophage T4 (T4) was purified as described previously[11,12]. The conjugation of goat anti-rabbit IgG IgG (anti-IgG) to T4 was based on the method of Avrameas[13] and modified as described previously[11,12]. The standard reaction mixture consisted of 5 ml of T4 (7 mg of protein/ml) and 0.5 ml

of IgG (35 mg of protein/ml) in phosphate buffer. The conjugation was carried out by freshly prepared glutaraldehyde in a final concentration of 0.05%. After 1 hour conjugation, glycine was added to a final concentration of 0.1M, to inactivate the remaining aldehyde groups. The mixture was dialysed overnight against phosphate buffer at 4°C and T4-anti-IgG conjugate (T4-anti-IgG) was collected by two cycles of alternate low- (at 2,000g, to remove aggregates) and high- (at 11,000 g, to separate T4-anti-IgG from free IgG molecules) speed centrifugations and used for scanning immunoelectron microscopic method.

Treatment of guinea pigs and preparation of specimens : Group 1. Guinea pigs were painted with total 0.4 ml of 5% DNCB-ethanol solution on shaved areas of both sides of inguinal regions.

Group 2. Animals were injected intraperitoneally with 250 mg/kg cyclophosphamide 3 days before painting with DNCB as described above.

Group 3. Animals were given two intraveneous injections of 2,4-dinitrobenzene sulfonic acid sodium salt ($DNBSO_3Na$) 600 mg/kg in each with a 14 days interval, the last dose being 14 days before the application of DNCB.

The draining lymph nodes were obtained from the guinea pigs at 12 hours after painting the skin with DNCB. Cell suspensions were prepared by teasing the nodes in a large amount of Eagle's minimal essential medium (MEM) containing 0.1% sodium azide (NaN_3) to exclude *in vitro* conjugation of cells with free DNCB remained in the nodes during the experimental processes. The cells were washed three times in Eagle's MEM containing NaN_3. One drop of the cell suspension was mounted on the surface of coverglass treated with poly-L-lysine, so that the cells could adhere on the glass in uniform distribution without losing their distinctive shape[14]. Subsequent reactions were carried out with cells adhering to the surface of poly-L-lysine coated coverslip.

Treatment of lymph node cells with anti-DNP and T4-anti-IgG : DNP cells were prefixed with 1% glutaraldehyde solution for 10 minutes and treated with 0.15M glycine and then washed three times in PBS (0.01M phosphate buffer saline, pH 7.2) containing NaN_3 and incubated with 50 μg/ml of anti-DNP in PBS at 37°C for 20 minutes. The specimens were then washed a further times in PBS and incubated with T4-anti-IgG in PBS (1mg/ml) at 37°C for 30 minutes and finally rinsed again with PBS[11,12].

For control experiments, non-conjugated T4, nonspecific goat IgG conjugated T4 and rabbit anti-human IgG IgG were used instead of T4-anti-IgG and anti-DNP, respectively. T4-anti-IgG was also used without preincubation of specific antibody (anti-DNP). Normal lymph node cells and the draining lymph node cells from guinea pigs painted with 4-ethoxymethylene-2-phenyl-5-oxazolone were treated stepwise with anti-DNP and T4-anti-IgG. The

experiments were also controlled by conventional blocking techniques using excess antibody (non-conjugated anti-IgG) and antigens (normal rabbit IgG and DNP-protein conjugates).

Preparation of samples for scanning electron microscopy: Samples were fixed with freshly prepared 2% glutaraldehyde solution in PBS for 60 minutes on ice, rinsed in PBS, and postfixed with 1% osmic acid. Dehydration was carried out in a graded series of ethanol (50-99%) and finally in two times of 100% ethanol[11,12]. After replacing ethanol with isoamylacetate, the critical point drying method using liquid carbon dioxide was applied. The specimens were coated with gold-palladium and observed with a field emission type scanning microscope (HS-2R, Hitachi Ltd).

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 examined by the indirect method of the scanning immunoelectron microscopy

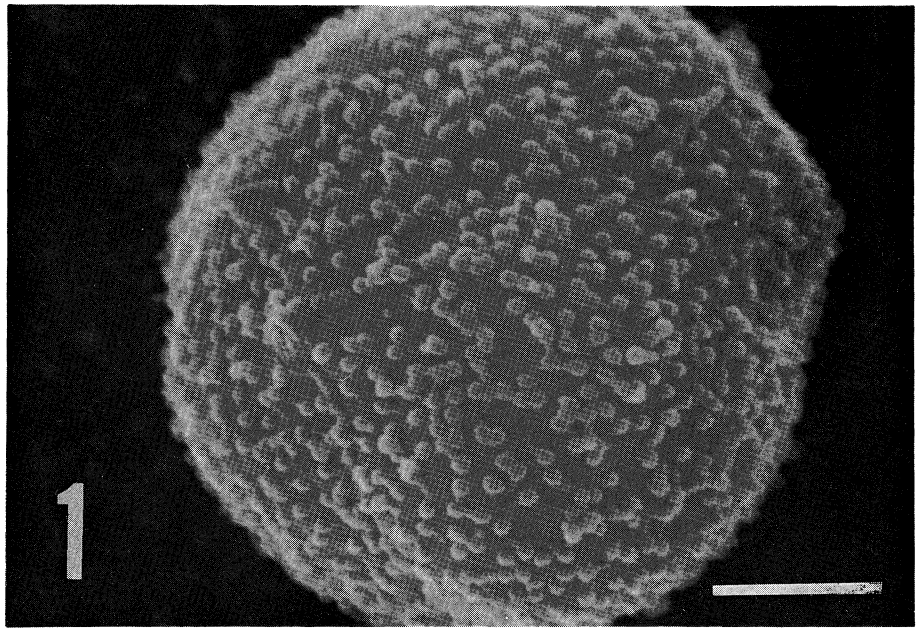
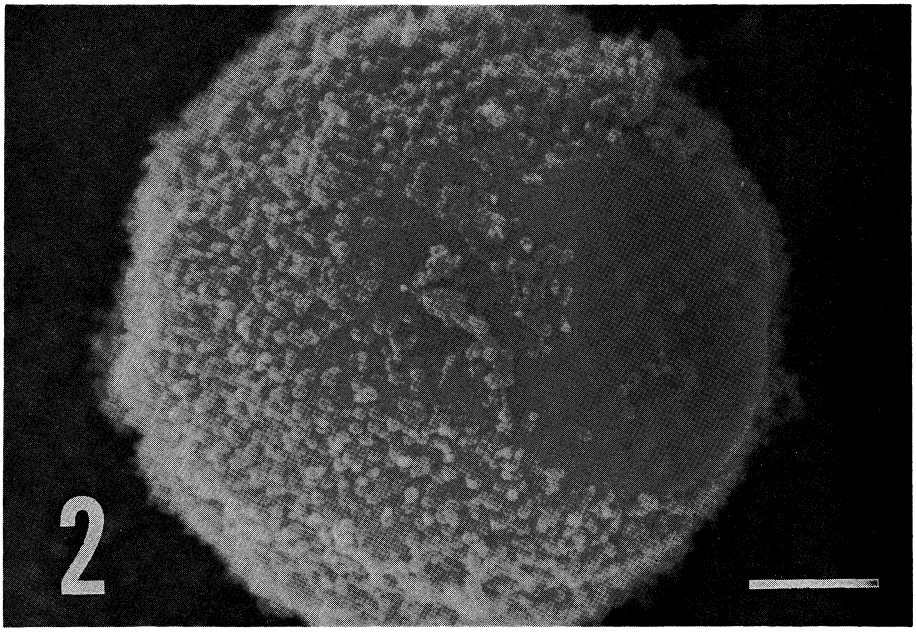
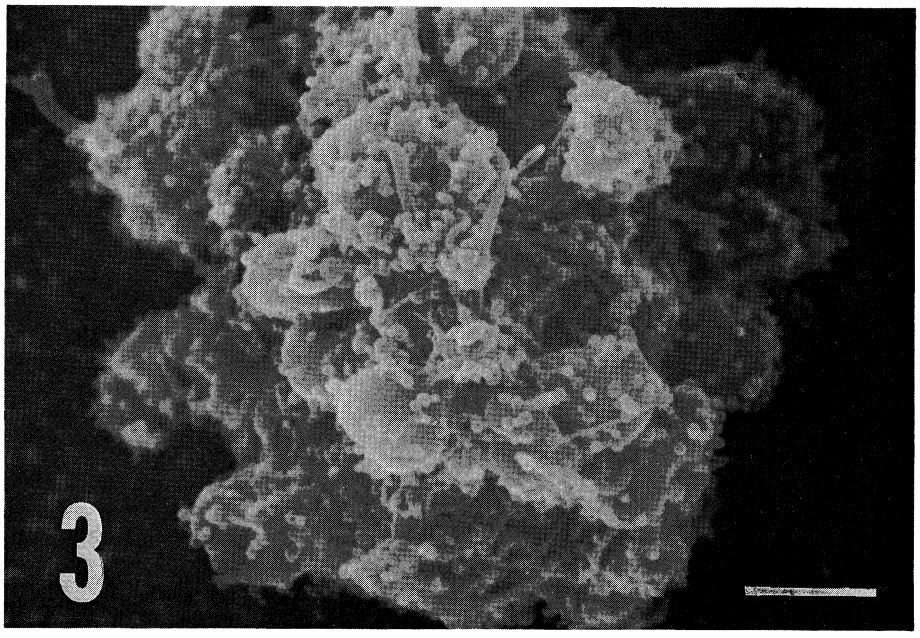


Fig. 1-3. Scanning electron micrographs of the cells in the draining lymph nodes taken from normal guinea pigs 12 hours after skin painting with DNCB, labelled by an indirect technique of scanning immunoelectron microscopy. The cells were treated with rabbit anti-DNP IgG, followed by goat anti-rabbit IgG antibody conjugated T4 bacteriophage. T4-anti-IgG are diffusely distributed over the cells. Bars=1 μm: x22,000, x16,000, x16,000.


2


3

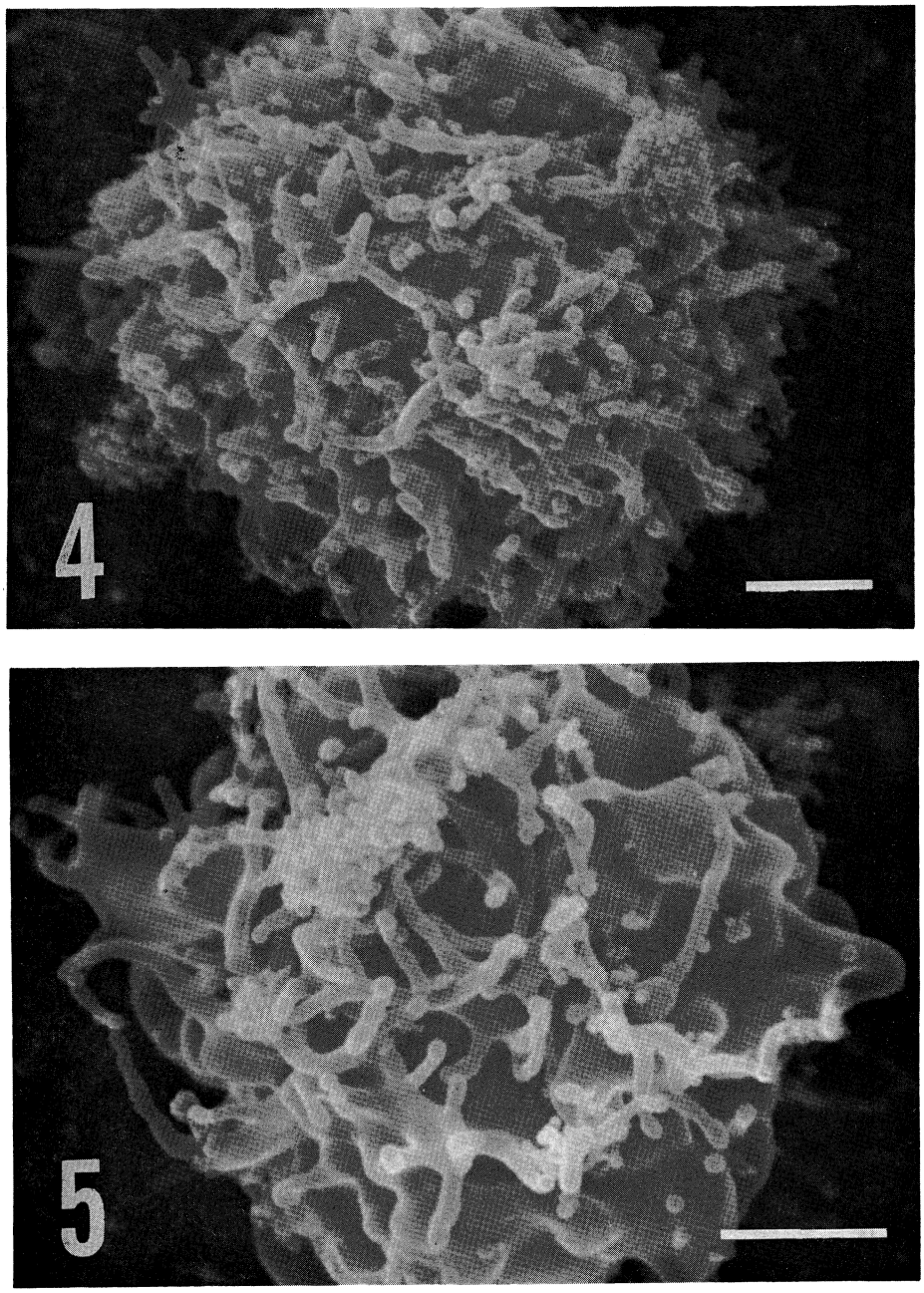
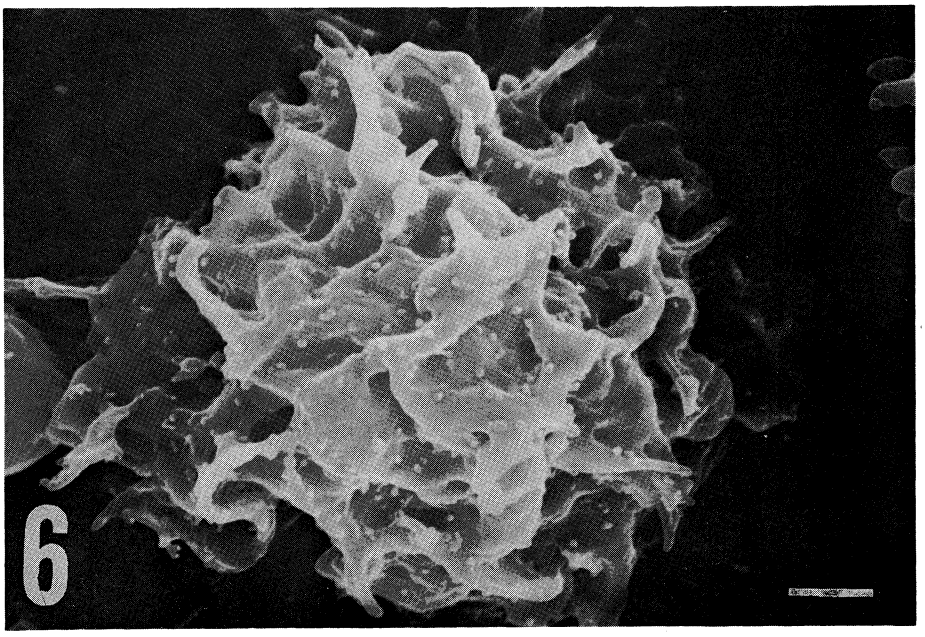


Fig. 4-5. The draining lymph node cells taken from normal guinea pigs 12 hours after painting with DNCB. T4 arranged in patchy distribution on the cells are observed. Bares=1 μm; x16,000, x22,000.



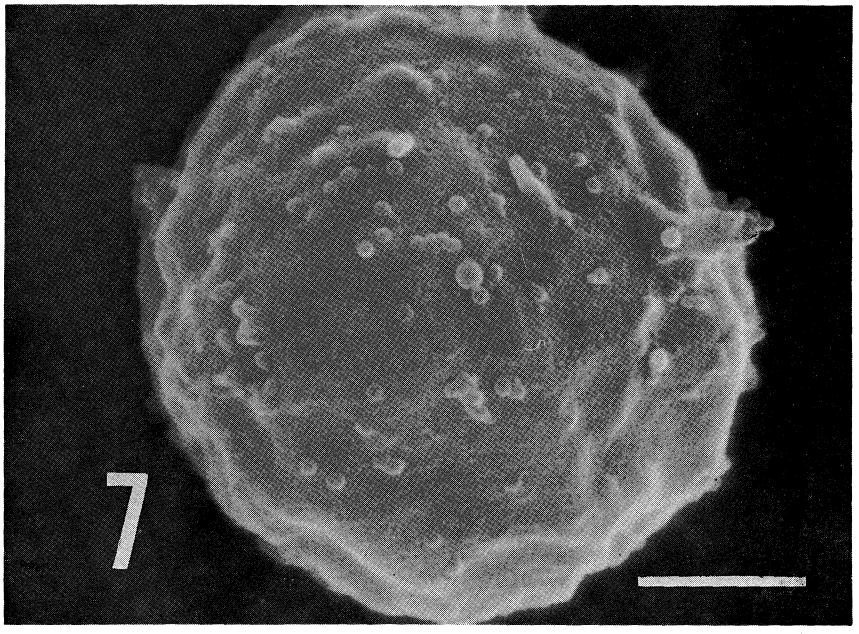


Fig. 6-7. The draining lymph node cells taken from normal guinea pigs 12 hours after painting with DNCB. The cells with sparse distribution of T4 anti-IgG are also seen. Bars=1 μm; x10,000, x22,000.

using rabbit anti-DNP IgG (anti-DNP) and becteriophage T4-goat anti-rabbit IgG IgG conjugate (T4-anti-IgG). As may be seen in Fig. 1-7, T4-anti-IgG were distributed over the treated lymph node cells. T4 labelling can be monitored easily at a relatively low magnification. The morphological identity of T4 was confirmed at a high magnification, because T4 can be more clearly resolved with the hexagonal head and the tail. The specificity of the labelling was checked and shown to be DNP specific (see Materials and Methods). Nonspecific adsorption of T4-anti-IgG to cell surface was hardly detected. As may be seen in these photographs, T4 were also viewed on the background. The adhesion of T4-anti-IgG may be dependent on the electric charges of the conjugate and of poly-L-lysine coated coverslip. It was confirmed in the control experiments that specificity of immunological reaction on the surface of the biological materials was not influenced by pretreatment of coverslip with poly-L-lysine. T4 labelling was classified roughly into three types, diffuse, patchy and sparse, as shown in Fig. 1-7. The incidences of the cells with

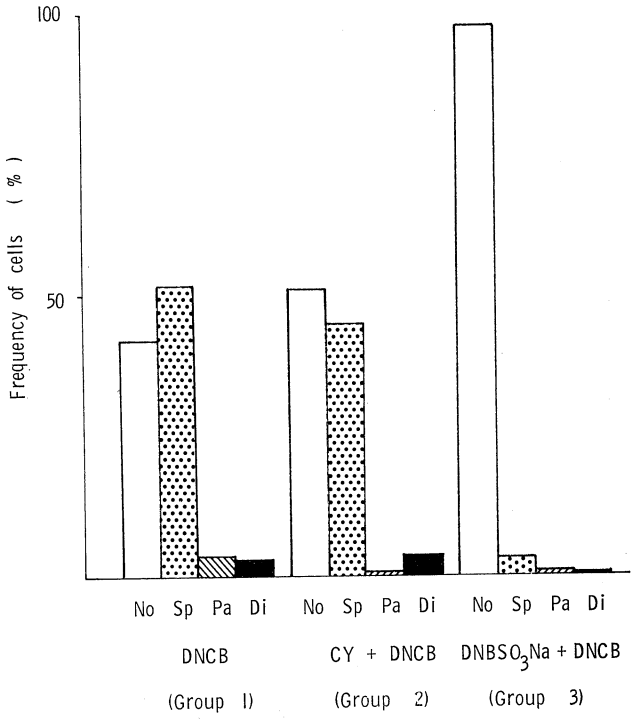


Fig. 8. Mean frequencies of the cells with various distribution patterns of T4 anti-IgG in the draining lymph nodes taken from normal and CY or $DNBSO_3Na$ treated guinea pigs 12 hours after skin painting with DNCB. Sp, sparse ; Pa, patchy ; Di, diffuse.

each type of T4 labellig in lymphnode cells were estimated (group 1 in Fig. 8.) The number of the cells tagged with T4 anti-IgG in different animals varied between 53% and 66%.

The effect of CY and tolerogen, $DNBSO_3Na$, on the distribution of DNP groups on lymph node cells was also studied. There was no fundamental difference in T4 labelling comparing animals given CY before painting with DNCB (group 2 in Fig. 8) with normal group which had simply been painted once with DNCB (group 1). The frequencies of the cells tagged with T4 anti-IgG were markedly reduced in the animals treated with $DNBSO_3Na$ (group 3) as compared to animals not receiving the tolerogen (group 1).

DISCUSSION

In our previous studies using the immunofluorescent method[6,7], DNP groups were found to be distributed on the cells in the draining lymph node of guinea pigs when DNCB was painted the skin. To investigate the distribution of DNP groups on the cells more closely, we used T4-bacteriophage labelled with antibody as visual marker so that DNP groups localization on the surface of the cells could be clearly followed with a scanning electron microscopy. Fairly precise localization of them could be mapped with T4. Based on T4 labelling, it is considered that DNP groups are distributed in various amounts and in various patterns on the surface of lymph node cells when DNCB is introduced percutaneously into body. The incidence of the cells on which DNP groups were detected was approximately 60% and was higher than that of the cells detectable by the immunofluorescent method[7]. The frequency of the cells with DNP groups were markedly reduced in the animals treated with $DNBSO_3Na$ (group 3), as compared to animals not receiving the tolerogen (group 1). It is suggested that the cells in the draining lymph node of guinea pigs treated with $DNBSO_3Na$ and then painted with DNCB release some factors which combine with DNP groups on the surface of lymph node cells, resulting in the decrease of the cells with DNP groups detectable by the scanning immuno-electron microscopic method by preventing the reaction of anti-DNP antibody with hapten groups on the cell surface[7]. The factors seem to be analogous to the so-called soluble suppressor factors which have been demonstrated in mice by Zembala and Asherson[15] and Moorhead[16]. On the other hand, the incidences of the cells with DNP groups were not fundamentally different in CY treated animals (group 2) when compared with animals which had not received CY (group 1). Previous work[7] has showed that difference in incidence of the cells with DNP groups in the draining lymph node comparing group 2 with control group 1 are not significant at 12 hours after painting with DNCB, but become significant at 24 hours.

One of the most important question raised by a study of this type is what is the function of the hapten cell complex in contact sensitivity induction. Cells taken from draining lymph nodes of mice 1 day after painting with contact sensitizers effectively induce contact sensitivity when injected into normal mice [17-19]. The induction occurs when there is genetic matching at the major histocompatibility complex between the donor and the recipient animal[20]. Contact sensitization is also achieved by injecting with lymph node cells of guinea pigs conjugated *in vitro* with sensitizers[1-3]. These evidences suggest that the hapten associated cells act as immunogens in the immunizing process of contact senitivity.

Acknowledgment

This work was supported by Kawasaki Medical School Grant No. 54-008 for project research.

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, Lond. **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 (in press)
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. and Claman, H. N.: Genetic restrictions for the induction of suppressor T cells by hapten-modified lymphoid cells in tolerance to 1-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) Aoshima, T., Nakagawa, S., Gotoh, M., Tanioku, K. and Amos, H. E.: Studies on the dinitrophenylated lymphocytes in guinea pig painted with DNCB. J. Dermat. **4**: 251-254, 1977
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) Fukushiro, S., Nakagawa, S., Gotoh, M., Koshizawa, M. and Tanioku, K.: The distribution of antigen in flare up reaction in contact sensitivity to DNCB. Immunology **34**: 549-553, 1978
9) 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 Dermat. **57**: 269-277, 1971
10) Eisen, H. N., Gray, W. R., Russell Little, J. and Sims, E. S.: In Methods in Immunology and Immunochemistry, Vol. 1 (Ed. by C. A. M. w. Chase), p. 355, Academic press Inc., New York, 1967
11) Kumon, H., Uno, F. and Tawara, J.: A morphologically recognizable marker for scanning immunoelectron microscopy. T4-bacteriophage. Virology **70**: 554-557, 1976
12) Kumon, H.: Morphologically recognizable markers for scanning immunoelectron

microscopy. II. An indirect method using T4 anb TMV. Virology **74**: 93-103, 1976
13) Avrameas, S.: Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugaton for the detection of antigens and antibodies. Immunochemistry **6**: 43-52, 1969
14) Tsutsui, K., Kumon, H., Ichikawa, H. and Tawara, J.: Preparative method for suspended biological materials for SEM by using of polycationic substance layer. J. Electron Microsc. **25**: 163-168, 1976
15) Zemabala, M. and Asherson, G. L.: T cell suppression of contact sensitivity in the mouse. II. The role of soluble suppressor factor and its interaction with macrophages. Europ. J. Immunol. **4**: 799-804, 1974
16) Moorhead, by W.: Soluble factor in the mouse I. Suppression of contact sensitivity J. soluble suppressor factor released in vitro by lymph node cell populations containing specific suppressor cells. J. Immunol. **119**: 315-321, 1977
17) 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**: 454-467, 1976
18) 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
19) 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
20) 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