Immunology 1979 **36** 851

The distribution of 2,4-dinitrophenyl groups in lymphoid tissue of guinea-pigs following skin painting with 2,4-dinitrochlorobenzene

S. NAKAGAWA, H. E. AMOS*, M. GOTOH & K. TANIOKU *Department of Dermatology, Kawasaki Medical School, Kurashiki, Japan and* **The British Industrial Biological Research Association, Carshalton, Surrey*

Received 24 July 1978; accepted for publication 5 October 1978

Summary. Cellular localization of 2,4-dinitrophenyl (DNP) groups in the peripheral lymphoid system of guinea-pigs was studied at various times after painting the skin with 2,4-dinitrochlorobenzene (DNCB) by the immunofluorescent method using anti-DNP antibody. The cells taking up the stain (DNP cells) were shown to be mainly lymphocytes. At 1–6 h after painting the majority of DNP cells were found in the peripheral blood and the spleen but the maximum number was reached in the lymph node draining the site of DNCB application 12 h after painting.

Injecting cyclophosphamide (CY) 3 days before painting with DNCB, heightened the number of DNP cells residing in the draining node. The animals treated with the tolerogen, 2,4-dinitrobenzene sulphonic acid sodium salt ($DNBSO_3Na$), and then painted with DNCB, had fewer DNP cells than those animals which had simply been painted once with DNCB. The culture supernatants prepared from the draining nodes of both normal and tolerant animals partially blocked the anti-DNP antibody binding with DNP cells.

It is suggested that the cells associated with DNP groups residing in the draining lymph node act as immunogens in the immunizing process of contact sensitivity.

Correspondence: Dr S. Nakagawa, Department of Dermatology, Kawasaki Medical School, 577 Matsushima, 701-01 Kurashiki, Japan.

0019-2805/79/0400-0851$02.00

INTRODUCTION

Despite much experimental effort by various workers on the induction of contact sensitivity, the mechanism is still unclear. The widely held concept that small molecular weight contactants must combine with skin proteins to form a complete antigen is unlikely to happen in every case of contact sensitization.

Recently a number of investigators have claimed that a contactant will attach to viable lymphocytes *in vitro* and that the combination forms an immunogenic unit (Baumgarten & Geczy, 1970; Polak & Macher, 1974; Jones & Amos, 1975). The conditions in which the *in vitro* coupling was carried out by these investigators was unphysiological, however, particularly with respect to the ratio of contactant concentration to cell number. This will inevitably be a point of contention as there are no quantitative data on the number and distribution of lymphoid cells taking up contact sensitizers *in vivo*.

The objects of the experiments reported in this paper were to obtain such quantitative data and to assess the effect of cyclophosphamide and tolerogen on the distribution of these cell contactant units in the animals that were treated percutaneously with a contact sensitizer.

MATERIALS AND METHODS

Animals

Dunkin-Hartley strain guinea-pigs weighing between 350–450 g were used throughout the experiments.

Production and characterization of anti-DNP antibody
Rabbits were injected with 4–8 mg of 2,4-dinitrophenyl $(DNP)_4$-ovalbumin at weekly intervals for 5 weeks. The first three injections were emulsified with Freund's complete adjuvant (Difco) and given into the footpads, subsequent injections were given subcutaneously. The animals were boosted after a 4 week rest period and then bled 7 days later. Small amounts of the antisera were tested by quantitative precipitation with DNP_8-bovine serum albumin (BSA) to determine the amount of antigen needed for optimum precipitation.

DNP_8-BSA was added to the antisera at equivalents and incubated for 1 h at 37° and for 24 h at 4°. The precipitate which formed was collected and washed with cold 0·15 M sodium chloride, then resuspended in 0·1 M 2,4-dinitrophenol, pH 7·5, for 1 h at 37° followed by 24 h at 4°. The mixture was centrifuged and the supernatant was applied to a double layer column of DEAE-cellulose and Dowex 1 (×8) 200–400 mesh (Sigma Chemicals Co., USA) as described by Eisen, Russell Little & Sims (1967). Saline 0·01 M potassium-phosphate was used as eluent.

The eluted anti-DNP antibody was characterized by gel diffusion against DNP-protein conjugates and by immunoelectrophoresis with goat anti-rabbit whole serum and goat anti-rabbit IgG.

Preparation of a fluorescence labelled anti-DNP antibody and assessment of cells bearing DNP groups (DNP cells)
A technique previously described by Nakagawa, Ueki & Tanioku (1971) was used. The labelled antibody had the following characteristics: fluorescein isothiocyanate (FITC) 30 μg/ml; total protein 7·5 mg/ml; fluorescein protein ratio 1·64 M or $4{\cdot}0 \times 10^{-3}$ weight. The conjugate was used after two absorptions with guinea-pig liver acetone powder and one absorption with normal guinea-pig leucocytes. This was used at a concentration of less than 50 μg specific antibody/ml.

Lymphoid cell smears were washed three times with PBS (0·01 M phosphate buffer saline, pH 7·2) and incubated with FITC-labelled anti-DNP antibody for 20 min at 37°. The specimens were then washed a further three times in PBS and mounted in glycerine. Florescence was examined with a Reichert Zetpan transmission microscope. The percentages of staining cells in a smear were determined by examination of the microscopic field in fluorescent light and conventional light alternately. Two to three thousand cells in a specimen were examined.

The experiments were controlled by conventional blocking techniques using excess unlabelled anti-DNP antibody and DNP-BSA, DNP-guinea-pig albumin conjugates.

Treatment of guinea-pigs
Group 1. Guinea-pigs were painted with 0·05 ml of a 5% DNCB-ethanol solution on a shaved area of the right inguinal region. The painted area was confined to a 30 mm diameter circle.

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

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

Group 4. Tolerance was induced by injections of $DNBSO_3Na$ as described, CY (250 mg/kg) was then injected 14 days after the last dose of the tolerogen and the animals were painted with DNCB 3 days later.

Preparation of lymphoid tissue cells
The lymphoid tissue and peripheral blood were obtained from the animals at various time intervals after painting the skin with DNCB. Cell suspensions were prepared by teasing the lymphoid tissue in Eagle's minimal essential medium (MEM). Blood leucocytes were separated by adding 1% methyl cellulose (Dow Chemicals Co., USA) to defibrinated blood. In some experiments, lymphocyte rich fractions were separated from lymph node cells by sodium metrozoate Ficoll (Nyegaard & Co., Norway) gradient centrifugation. More than 97% of the cells were considered to be lymphocytes by morphological criteria. The cells prepared from lymphoid tissue and blood were washed three times with PBS, and then smeared, air dried and fixed in 95% ethanol for 30 min.

In vitro preparation of lymph node cells bearing DNP groups
A lymphocyte rich fraction of lymph node cells taken from normal guinea-pigs was incubated with 2·5 mM $DNBSO_3Na$ in PBS (2×10^7 cells/ml) for 60 min at 37°. The cells were then centrifuged and thoroughly washed in PBS.

Preparation of lymph node cell culture supernatants and treatment of the in vitro prepared DNP cells
Culture supernatants were prepared from the four

groups of animals described above. Normal animals and the animals which had been injected with CY or $DNBSO_3Na$ were painted with DNCB. The lymphocyte rich fractions were prepared from the nodes draining the site of application and from the contralateral equivalents, 1, 12 and 24 h post-painting. The cells were cultured in Eagle's MEM 10% foetal calf serum, penicillin, streptomycin and glutamine for 48 h at 37° as described by Zembala & Asherson (1974). The gas phase was CO_2 5%; air 95%. The supernatants were collected by centrifugation at 4200 *g* for 30 min. The supernatants which were derived from the animals treated as above, but without the painting dose of DNCB, were also prepared. The DNP cells prepared *in vitro* were incubated in the supernatants for 1 h at 37° (10^7 cells/ml), and subsequently DNP cells were assessed by the immunofluorescent method.

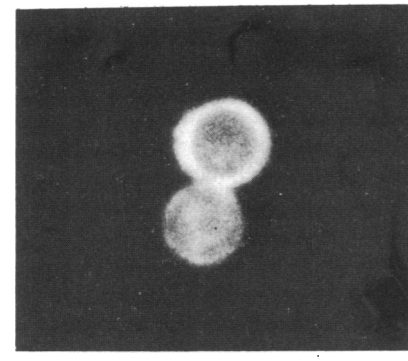

Figure 1. Lymphocytes in the lymph node draining site of DNCB application 12 h after painting stained by the direct immunofluorescent method for DNP groups. Specific staining in peripheral or homogeneous patterns is noted (smear, original magnification × 200).

RESULTS

Data relating to the number of cells in the peripheral lymphoid tissue of DNCB painted guinea-pigs (group 1) that react with anti-DNP antibody are given in Table 1. The cells were counted at different time intervals after painting with DNCB. Specificity of the fluor-

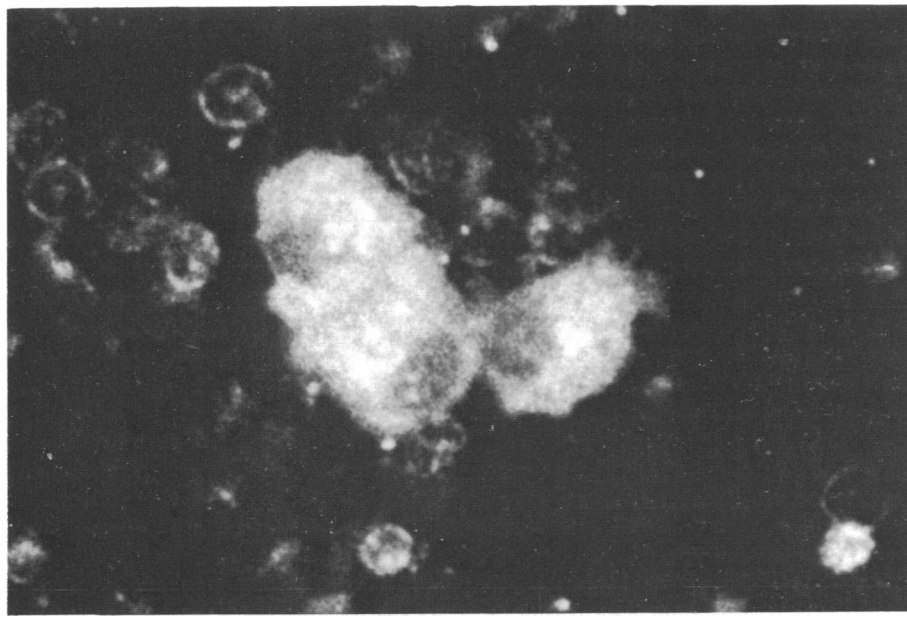

Figure 2. Macrophages or reticulum cells in the draining node 24 h after painting are also stained. Note specific staining in the areas corresponding to the cytoplasm of the cells (tissue imprint, original magnification × 200).

Table 1. Distribution of DNP cells in lymphoid tissue of normal guinea-pigs at various times after painting with DNCB to the right inguinal skin

Tissue	Frequencies* of DNP cells (%)								
	1 h†	6 h	12 h	18 h	1 day	2 days	3 days	4 days	7 days
Right inguinal node	0·05 (0·02–0·07)	0·31 (0·25–0·34)	0·45 (0·38–0·48)	0·22 (0·19–0·25)	0·08 (0·02–0·11)	0·02 (<0·01–0·05)	<0·01	<0·01	<0·01
Left inguinal node	<0·01	<0·01	<0·01	<0·01					
Mesenteric node	<0·01	<0·01	<0·01	<0·01					
Thymus	<0·01	<0·01	<0·01		No DNP cell				
Spleen	0·19 (0·07–0·34)	0·08 (0·07–0·08)	0·03 (0·01–0·04)	<0·01					
Peripheral blood	0·21 (0·10–0·32)	0·25 (0·21–0·28)	0·05 (0·03–0·06)	0·01 (<0·01–0·02)					

* Mean percentages and ranges of DNP cells from five animals.
† Time after skin painting with DNCB.

Table 2. Distribution of DNP cells in the lymph node draining site of skin painting with DNCB in CY or $DNBSO_3Na$ treated guinea-pigs

Animal group	Experimental protocol			Frequencies* of DNP cells (%)		
				1 h†	12 h	24 h
1			DNCB	0·12 (0·01–0·33)	0·61 (0·52–0·89)	0·08 (0–0·28)
2		CY	DNCB	0·28 (0·21–0·41)	0·94 (0·72–1·02)	0·67 (0·40–0·94)
3	$DNBSO_3Na$		DNCB	0·09 (0·02–0·17)	0·08 (0–0·21)	0·00
4	$DNBSO_3Na$	CY	DNCB	ND	0·46 (0·20–0·67)	0·29 (0·21–0·34)

* Mean percentages and ranges of DNP cells from five animals.
† Time after skin painting with DNCB.
ND, not done.

escent staining was checked and shown to be DNP specific; the cells taking up the stain were shown by counter staining to be mainly lymphocytes and a small population of macrophages or reticulum cells (Figs 1 and 2).

It can be seen from Table 1 that of the total cell complement only a small percentage reacted with the antibody. At 1–6 h after painting the majority of reacting cells (DNP cells) were found in the peripheral blood and the spleen but the maximum number was reached in the node draining the site of DNCB application 12 h after painting. At 3 days few DNP cells were found in any of the lymphoid compartments.

In the following experiments, DNP cells in the lymphocyte rich fraction of the draining nodes were counted. The percentages of the DNP cells in animals of group 1 (Table 2) were higher than those of the experiment described above in which all populations of lymph node cells except erythrocytes were examined (Table 1). Injecting CY 3 days before painting with DNCB, heightened and prolonged the number of DNP cells residing in the gland (group 2 in Table 2).

Table 3. Frequencies of *in vitro* prepared DNP cells after incubating with culture supernatants prepared from lymph node cells of guinea-pigs following skin painting with DNCB

Animal groups	Source of culture cells: Treatment of animals*				Lymph nodes	Frequencies of *in vitro* DNP cells after incubation with supernatants (%)	
	Control					4·0†	
		Nil			Inguinal	4·0	
1		DNCB	(1 h‡)		Draining	3·8	4·0§
					Contralateral	4·0	
		DNCB	(12 h‡)		Draining	2·9	3·8§
					Contralateral	4·1	
		DNCB	(24 h‡)		Draining	2·7	4·0§
					Contralateral	3·9	
2		CY DNCB	(12 h‡)		Draining	3·9	
	$DNBSO_3Na$	Nil			Inguinal	3·9	
3	$DNBSO_3Na$	DNCB	(1 h‡)		Draining	3·2	3·8§
					Contralateral	3·7	
	$DNBSO_3Na$	DNCB	(12 h‡)		Draining	2·2	3·9§
					Contralateral	3·6	
	$DNBSO_3Na$	DNCB	(24 h‡)		Draining	2·2	4·0§
					Contralateral	3·7	
4	$DNBSO_3Na$	CY DNCB	(12 h‡)		Draining	3·4	

* Three animals were used in each experiment.
† Percentage of *in vitro* prepared DNP cells before incubation with supernatants.
‡ Time after skin painting with DNCB.
§ Percentages of DNP cells after incubation with the supernatants neutralized with DNP–ovalbumin conjugate.

Guinea-pigs treated with the tolerogen $DNBSO_3Na$ and then painted with DNCB, had fewer DNP cells (group 3) than those animals which had simply been painted once with DNCB (group 1). DNP cells did, however, appear in the tolerized animals if they were injected with CY before painting with DNCB (group 4).

The inability to demonstrate DNP cells in the tolerized group of animals even after application of DNCB, was investigated further by attempting to demonstrate a soluble blocking factor(s) which prevented the FITC labelled anti-DNP antibody reacting with any DNP cells which might be present in the draining node. The basis of the experiment was to culture lymph node cells from guinea-pigs treated in various ways (see Materials and Methods), and then incubated DNP cells prepared *in vitro* with the different cell free supernatants. The typical results obtained from one of five separate experiments are presented in Table 3.

It can been seen that culture supernatants prepared from the draining node of both tolerant and normal animals (groups 1 and 3) partially blocked the anti-DNP antibody uptake. This was most evident if the supernatants were prepared from cells taken 12–24 h after painting, and abrogated by CY (groups 2 and 4). The supernatants prepared from the contralateral nodes completely failed to inhibit the antibody uptake. The supernatants prepared from the inguinal lymph nodes of tolerant and normal guinea-pigs without the painting dose of DNCB also failed to block the antibody binding. The inhibitory factors present in the supernatants were shown to be specific for DNP since they could be neutralized by incubating the supernatants with a DNP-ovalbumin conjugate (2 mg/ml) for 30 min at 37° before reacting with anti-DNP antibody.

DISCUSSION

The data presented in this paper showed that within the lymph node draining a site of DNCB application a small population of cells was present with DNP groups on them. The studies using a scanning immunoelectromicroscope method have demonstrated that the DNP groups are localized on the surface of either lymphocytes, both T and B cells, or macro-

phages (Gotoh, Nakagawa & Kumon, in preparation). Regarding the mechanism of surface binding of DNP groups to lymphocytes, it is possible to offer two explanations, which fit the data: (1) DNCB reacts directly *in vivo* with the protein constituents of the cell membrane; and (2) binding is dependent on the presence of an antigen-binding-receptor for DNP groups which was demonstrated on lymphocytes by Davie & Paul (1971). They reported that normal guinea-pigs possess a population of lymphocytes which bind DNP-homologous albumin conjugate to their surface in amounts detectable by radioautography. Demonstration of *in vivo* and *in vitro* binding of DNP-guinea-pig serum conjugate to the lymph node cells of guinea-pigs is, however, unsuccessful by the immunofluorescent method (Aoshima, Nakagawa, Gotoh, Tanikou & Amos, 1977). In addition, it has been shown in that paper that DNCB in free form remains in thoracic duct lymph at least for 12 h post-painting. These findings provide support for the first possibility described above.

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 (Asherson & Mayhew, 1976; Asherson, Zembala & Mayhew, 1977; Thomas, Asherson & Perera, 1978). Contact sensitization is also achieved by injecting with lymphoid cells of guinea-pigs conjugated *in vitro* with sensitisers (Baumgarten & Geczy, 1970; McFarlin & Balfour, 1973; Polak & Macher, 1974; Jones & Amos, 1975). This evidence suggests that the hapten associated cells act as immunogens in the immunizing process of contact sensitivity.

The DNP cells could be increased by treating the animals with CY before painting with DNCB. On the other hand, tolerizing the animals with $DNBSO_3Na$ reduced the number of DNP cells in the node, but they could be increased again by an injection of CY. The experiments using DNP cells prepared *in vitro* clearly show that culture supernatants from tolerized or normal guinea-pigs possess specific DNP factors which can effectively block the anti-DNP antibody reacting with DNP cells. The factors possibly cause reduction in the number of detectable DNP cells in the draining lymph nodes of normal and tolerized animals. The pretreatment with CY is considered to prevent the production of the factors.

The factors taken from the tolerized animals seem to be analogous to the so-called soluble suppressor factors which have been demonstrated in mice by Zembala & Asherson (1974) and Moorhead (1977). In the dinitrobenzene system, it has been shown that the development of tolerance by treating with tolerogen is associated with the reduction of immunoblast proliferation in the lymph node draining the site of contact sensitizer painting (Polak & Turk, 1974; Moorhead, 1976; Parker, Turk & Scheper, 1976). It is possible to suggest that the factors interfere with the development of early stages in the afferent limb of sensitization in the draining node by masking DNP groups on the lymph node cells and then preventing the recognition of the groups by immunologically competent cells. Of interest is that similar activity could be demonstrated in the culture supernatants of the draining lymph node cells from normal guinea-pigs following skin painting with DNCB. At the present moment, it is not possible to be more explicit about the nature of the factors and their role in the inductive phase of contact sensitivity. Clearly more needs to be done in this experimental area.

ACKNOWLEDGMENTS

We should like to thank the Herbert Duhill Trust for financial support for one of us (S. N.). This study was supported in part by a grant from the Japanese Ministry of Education (No. 257385).

REFERENCES

AOSHIMA T., NAKAGAWA S., GOTOH M., TANIOKU K. & AMOS H.E. (1977) Studies on the dinitrophenylated lymphocytes in guinea-pig painted with DNCB. *J. Dermatol.* **4,** 251.

ASHERSON G.L. & MAYHEW B. (1976) 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.

ASHERSON G.L., ZEMBALA M. & MAYHEW B. (1977) 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.

BAUMGARTEN A. & GECZY A.F. (1970) Induction of delayed hypersensitivity by dinitrophenylated lymphocytes. *Immunology,* **19,** 205.

DAVIE J.M. & PAUL W.E. (1971) Receptors on immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea-pig albumin–^{125}I-binding lymphocytes of normal guinea-pigs. *J. exp. Med.* **134,** 495.

EISEN H.N., GRAY W., RUSSELL LITTLE J. & SIMS E.S. (1967) In: *Methods in Immunology and Immunochemistry*, Vol. 1 (Ed. by C. A. Williams and M. W. Chase), p. 355. Academic Press Inc., New York.

JONES J.M. & AMOS H.E. (1975) Antigen formation in metal contact sensitivity. *Nature (Lond.)*, **256,** 499.

MCFARLIN D.E. & BALFOUR B. (1973) Contact sensitivity in the pig. *Immunology*, **25,** 995.

MOORHEAD J.W. (1976) Tolerance and contact sensitivity to DNFB in mice. VI. Inhibition of afferent sensitivity by suppressor T cells in adoptive tolerance. *J. Immunol.* **117,** 802.

MOORHEAD J.W. (1977) Soluble factor in tolerance and contact sensitivity to 2,4-dinitrofluorobenzene in mice. I. Suppression of contact sensitivity by soluble suppressor factor released *in vitro* by lymph node cell populations containing specific suppressor cells. *J. Immunol.* **119,** 315.

NAKAGAWA S., UEKI H. & TANIOKU K. (1971) 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.

PARKER D., TURK J.L. & SCHEPER R.J. (1976) Central and peripheral action of suppressor cells in contact sensitivity in the guinea-pig. *Immunology*, **30,** 593.

POLAK L. & MACHER E. (1974) *In vitro* sensitization of dinitrochlorobenzene in guinea-pigs. *Nature (Lond.)*, **252,** 748.

POLAK L. & TURK J.L. (1974) Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. *Nature (Lond.)*, **249,** 654.

THOMAS W.R., ASHERSON G.L. & PERERA M.A.C.C. (1978) 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.

ZEMBALA M. & ASHERSON G.L. (1974) 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.