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BRIEF NOTE

THE EFFECT OF CYCLOPHOSPHAMIDE AND TOLEROGEN ON THE DISTRIBUTION OF ANTIGEN IN CONTACT SENSITIVITY

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Abstract

Pretreatment of guinea pigs with CY caused more frequent DNP lymphoid cells in the lymph node draining the site of painting with DNCB, when compared with those of normal animals. On the other hand, injection of animals with $DNBSO_3Na$ resulted in reduction in frequencies of DNP cells.

2, 4-Dinitrophenyl (DNP) groups have been found attached mainly to lymphocytes throughout the peripheral lymphoid system of guinea pigs following painting the skin with 2, 4-dinitrochlorobenzene (DNCB) by the immunofluorescent method using the antibody against DNP groups[1]. Evidence has also been shown which indicates that DNCB penetrates through the skin and reacts directly with the cell membrane of the cells in the lymphoid system[3]. An analysis of the number of the dinitrophenylated (DNP) cells in the lymphoid system at different time intervals after painting has showed that the incidence of such cells is high, particularly in the lymph nodes draining the site of such painting with DNCB, and that it is maximal at 12 h and declines quickly after that[1,3]. We should like to report that the DNP cells detectable by the immunofluorescent method vary in number according to whether treating the guinea pigs with cyclophosphamide (CY) or tolerogen before painting the skin with DNCB.

The guinea pigs used were of the male Hartley strain (350-450 g). Animals were injected intraperitoneally with 250 mg/kg CY 3 d before sensitization. Tolerance was induced by two intraveneous injections of 600 mg/kg 2, 4-dinitrobenzene sulfonic acid sodium salt ($DNBSO_3Na$) in each with an interval of 14 d, the last dose being 14 d before an attempt at sensitization. For sensitization of the animals, an application of 0.05 ml of 5 % DNCB-ethanol solution was given to the inguinal skin. Sensitivity was tested by contact with 0.2, 0.09, 0.05 and 0.01 % DNCB in ethanol on the flank 7 d later. The intensities of skin reactions were assessed 24 h later as described

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previously[2]. Inguinal lymph nodes draining the site of application of a sensitizing dose of DNCB and peripheral blood were obtained 1, 12 and 24 h after the administration of DNCB. Lymphocyte rich fractions were separated from the lymph nodes and peripheral blood by sodium metrizoate Ficoll gradient centrifugation and washed twice in PBS (0.01 M phosphate buffer saline, pH 7.2). More than 97 % of the cells from lymph node and 93 % of the cells from blood were considered to be lymphocytes by morphologic criteria. DNP cells were detected by the immuofluorescent method using fluorescein isothiocyanate labelled antibody to DNP groups as described previously[1,3]. The percentage of the stained cells was determined by examination of the microscopic field in fluorescent light and conventional light alternately.

Table 1 shows that CY given 3 d before sensitizing procedure (group 2) caused more frequent DNP cells in the regional lymph node and the peripheral blood when compared with those frequencies of animals which had not received CY (group 1). In contrast, the frequencies of DNP cells were markedly reduced in the animals treated with $DNBSO_3Na$ (group 3) as compared to animals not receiving the tolerogen (group 1). The frequencies of DNP cells in regional lymph nodes and peripheral blood correlated well with the intensities of skin test reactions in these animal groups.

CY was given intraperitoneally to the unresponsive animals 3 d before sensitization, i. e. 14 d after the last dose of tolerogen (group 4). The injection of CY effectively reversed the unresponsiveness of the guinea pigs to DNCB as described by Polak and Turk[4]. The reversal of the unresponsiveness was associated with the increase of the frequencies of DNP cells in regional nodes and blood. The unresponsive animals in the other animal group (group 5) were painted with DNCB on the right inguinal skin 14 d after the last dose of tolerogen. CY was injected intraperitoneally to the animals 2 d later and then DNCB was applied to the other side of inguinal skin 3 d after the injection of CY. DNP cells from the lymph node draining the site of the latter application of DNCB and blood were assessed. It can be seen that painting with DNCB before CY treatment inhibits the reversals of either the unresponsiveness to DNCB or the frequencies of the detectable DNP cells in regional nodes and blood. Correlations of frequencies of DNP cells in regional nodes and blood with the intensities of skin reactivities were also observed in the animals of group 4 and 5.

It has been shown by Zembala and Asherson[5] that the lymph node cells from mice injected with picryl sulfonic acid liberate suppressor factor *in vitro* when exposed to picryl chlorid. These factors can specifically inhibit the passive transfer of contact sensitivity by immune lymph node cells and can be

TABLE 1 Frequencies of DNP cells in regional lymph nodes and peripheral blood of CY or $DNBSO_3Na$ treated guinea pigs following painting skin with DNCB

Animal groups	Experimental protocol	Tissue / Time after painting (h)	Mean frequencies of DNP cells (%)						Mean skin reactivities
		Tissue	Regional lymph nodes			Peripheral blood			
		Time after painting (h)	1	12	24	1	12	24	
1	DNCB		0.22(5)	1.07(9)	0.13(8)	0.22(5)	0.10(9)	0 (8)	2.0(6)
2	CY DNCB		0.38(3)	1.46(8)	0.99(7)	0.43(3)	0.23(8)	0.15(7)	3.0(6)
3	$DNBSO_3Na$ DNCB		0.21(5)	0.17(5)	0 (7)	0.10(5)	0 (5)	0 (7)	0 (5)
4	$DNBSO_3Na$ CY DNCB			0.58(3)	0.38(3)		0.07(3)	0.07(3)	1.0(5)
5	$DNBSO_3Na$ DNCB CY DNCB			0.01(4)	0 (3)		0 (4)	0 (3)	0.3(5)

The figures in parentheses refer to the numer of animals in each experiment.

adsorbed on to and eluted from picrylated albumin bound to sepharose. Next experiments were carried out to determine whether the lymph node cells from the guinea pigs release similar factors *in vitro* on exposure to DNCB. Guinea pigs were treated with $DNBSO_3Na$ as described above. The unresponsive and intact animals were painted with 0.05 ml 5 % DNCB on the inguinal skin 1, 12 or 24 h before harvesting the lymph node cells (q. v. group 3 and 1 in Table 1). The draining and contralateral lymph nodes were taken, and the lymphocyte rich fractions were separated from the nodes by sodium metrizoate Ficoll gradience and were cultured in Eagle's minimal essential medium containing 10 % foetal calf serum, penicillin, strepomycin and glutamine at 37°C for 48 h, as described by Zembala and Asherson[5]. The gas phase was CO_2 5 %; air 95 %. The supernatants were collected by centrifugation at 4,200 G for 30 min. Control supernatants were prepared from the unresponsive and intact animals which had not been painted with DNCB before harvesting lymphocytes. *In vitro* dinitrophenylated cells were prepared by incubation of lymphocyte rich fraction of lymph node cells from normal animals in 2.5 mM $DNBSO_3Na$ in PBS (2×10^7 cells/ml) at 37°C for 1 h, spun down and washed sufficiently. The DNP cells prepared *in vitro* were incubated in the supernatants at 37°C for 1 h, and subsequent DNP cells were assessed by the immunofluorescent method as described above. Table 2 shows typical results

TABLE 2 Frequencies of DNP cells incubated with culture supernatants of lymph node cells from unresponsive and intact animals

Incubated with culture supernatants of	Frequencies of DNP cells (%)			
	Unresponsive animals		Intact animals	
No incubation	4.1		4.0	
Animals* without DNCB painting, inguinal lymph nodes	3.9		4.0	
Animals* painted with DNCB (1h†), regional lymph nodes	3.2	3.8 §	3.8	4.0 §
contralateral lymph nodes	3.7		4.0	
Animals* painted with DNCB (12h†), regional lymph nodes	2.2	3.9 §	2.9	3.8 §
contralateral lymph nodes	3.6		4.1	
Animals* painted with DNCB (24h†), regional lymph nodes	2.2	4 0 §	2.7	4.0 §
contralateral lymph nodes	3.7		3.9	
CY treated animals* painted with DNCB (12h†), regional lymph nodes	3.4		3.9	

* Two to three unresponsive or intact animals were used.
† Lymph nodes were obtained at the time after painting with DNCB.
§ The frequencies of DNP cells after absorption of the supernatants with DNP-ovalbumin.

obtained from one of five separate experiments. Expectedly, the incubation of *in vitro* prepared DNP cells in unresponsive supernatants (i. e. the supernatant from the regional lymph node of unresponsive animal, 10^7 cells/ml) caused less frequent DNP cells detectable by the immunofluorescent procedure as compared to control supernatant. On the other hand, the frequencies of DNP cells also decreased when incubated in the supernatants from regional nodes which were taken from intact animals 12 and 24 h after painting with DNCB (intact supernatant). However, intact supernatants obtained 1 h after exposure to DNCB did not produce a reduction in the detectable DNP cells. The activities of the unresponsive and intact supernatants disappeared after being incubated with DNP-ovalbumin (2 mg/ml) at 37°C for 30 min. The frequencies of DNP cells which were incubated in the supernatants from contralateral lymph nodes of the unresponsive and intact animals were not significantly reduced. The intraperitoneal injection of CY 3 d before painting with DNCB on intact animals (q. v. group 2) was associated with a complete return of frequencies of DNP cells to the level in control supernatants. Treatment of unresponsive animals with CY (q. v. group 4) also resulted in a considerable rise in the frequencies of DNP cells. It was shown through the five separate experiments that the decrease in frequencies of DNP cells detectable by immunorescent method was larger when incubating *in vitro* DNP cells with unresponsive supernatants than when incubating in intact supernatants.

Then *in vitro* systems indicate the regional lymph node cells from the guinea pigs injected with $DNBSO_3Na$ and then painted with DNCB liberate some factor(s) *in vitro* which combine with DNP groups on the surface of lymphoid cells, resulting in the decrease of DNP cells detectable by immunofluorescent method by preventing the reaction of fluorescent antibody with DNP groups. It is reasonable to assume that such factors are also released *in vivo* and thereby cause the reduction in the number of DNP cells in the regional lymph node and peripheral blood of the unresponsive animals. This also suggests that the factors interferes with the development of the early stages in afferent limb of sensitization in regional lymph node by masking DNP groups on lymphoid cells and then preventing the recognition of the groups by lymphocytes. There is evidence that the development of unresponsiveness in guinea pigs by treatment with $DNBSO_3Na$ is associated with a marked reduction in the number of immunoblasts in the regional lymph node seen 4 d after sensitization with DNCB[4]. It has also been demonstrated that cell proliferation measured by DNA synthesis and number of immunoblasts in the draining lymph nodes of recipients of suppressor cells after sensitization with dinitrofluorobenzene are significantly less than in control mice and guinea pigs[6,7].

It was found that the regional lymph node cells from the intact guinea pigs on exposure to CNCB also released *in vitro* the factor(s) which cause DNP cells detectable by the immunofluorescent method to be reduced in number. Treatment of the animals with CY 3 d before being painted with DNCB inhibited the liberation of the factors *in vitro* and simultaneously caused more frequent DNP cells in regional lymph nodes and peripheral blood when compared with those frequencies in intact animals. A possible explanation of these findings is that during normal sensitization with DNCB, the lymphoid cells which produce the factors proliferate in the regional lymph nodes, and CY pretreatment depletes the population of the cells. Further investigations are necessary to clarify the character of the factors and their participation in the development of contact sensitivity.

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