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Model for Evaluating Cross-Sensitivity of DNBS with DNCB Using Hapten-Stimulated *In Vitro* Interleukin-2 Production by Murine Lymph Node Cells

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Prediction of cross-sensitivity among environmental chemicals has recently received considerable attention. Like identification of contact sensitizers, in vivo methods such as the mouse ear swelling test and the guinea pig maximization test are generally employed (Gad et al. 1986; Klecak 1987; Karlber et al. 1994). Some drawbacks adherent to these methods have been pointed out. They are subjective, time-consuming, lack sensitivity and require a large numbers of animals. To overcome these problems in in vivo tests, the development of suitable in vitro methods are expected. Recently, various efforts have been made to evaluate the cross-sensitivity using hapten-stimulated lymphocyte blastogenesis test (Frosch et al. 1989; Blasi et al. 1993). In our previous study (Xu et al. 1993), it has proven that hapten (DNCB)-stimulated in vitro IL-2 production by lymph node cells from DNCB-sensitized mice was dose-dependent and was positively related with in vivo contact hypersensitivity, suggesting that the in vitro IL-2 production may evaluate the contact sensitizing potentials of chemicals. Therefore, the present study was aimed at detecting cross-sensitivity of DNBS with DNCB using hapten-induced in vitro IL-2 production by the lymph node cells.

MATERIALS AND METHODS

Female Balb/c mice, at 8–12 weeks of age, were purchased from Shizuoka Animal Center, Japan. Six age-matched mice per group were used. Mice were maintained in laminar flow cabinets and provided sterile food and water *ad libitum*. DNCB (1-chloro-2,4-dinitrobenzene, Wako Chem Ind, Japan) and DNBS (2,4-dinitrobenzene sulphonic acid, Tokyo Kasei Ind, Japan) were used as contact sensitizers. DNCB and DNBS for in vivo use were dissolved in acetone/olive (4:1) and distilled water, respectively, whereas DNBS for in vitro assays was directly prepared in RPMI-1640 medium.

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Mice were shaved on the abdomen. 100 microliters of the freshly prepared 0.5% DNCB, 2.5% DNCB and acetone/olive, respectively, were applied to the shaved areas. In 6 days, The right and left ears of mice were challenged by one topical application of 25 microliters of 1% DNBS and equal values of acetone/olive, respectively. Before challenge and 24 hr thereafter, triplicate measurements of ear thickness were made using dial thickness gauge. Ear swelling response was presented as percent increase in ear thickness.

After measurements of ear thickness, mice were sacrificed to remove the inguinal, axillary and subscapular lymph node cells and were pooled for individual mouse. A single cell suspension of lymph node cells was prepared by routine method and resuspended in RPMI-complete medium. The viability of the cells used for in vitro assays was more than 90%. For in vitro induction of IL-2, the lymph node cells at $5x10^5$ cells/well were cultured with the indicated concentration of hapten or medium along in 200 microliter of RPMI-complete medium in U-shaped microwell plates at 37°C in a 5% CO_2 incubator. 48 hr thereafter, microplates were centrifuged at 1,500 rpm for 15 minutes. Then, the supernatant fluids were collected, frozen at -20°C for IL-2 assay.

IL-2 in the supernatants was measured by its ability to stimulate IL-2-dependent CTLL-2 cell growths (Ricken Cell Bank,Tsukuba, Ibaraki Prefecture, Japan) as assessed by 3-(4,5-dimethylthiazolzyl)-2,5-diphenyltetrazolium (MTT) colorimetric assay (Tada et al. 1986). In brief, 50 microliter of $8x10^5$ cells/ml were added to the flat-bottomed microwells containing 50 microliters of the supernatant samples and cultured at 37°C for 24 hr in a 5% CO_2 incubator. 20 microliters of MTT solution (5 mg/ ml in PBS) were then added to each well. After cultured for another 4 hr, 100 microliters of 10% SDS-0.01 N chloric acid solution were used to terminate reaction and the microplates were reincubated overnight. Optical density were measured on a microplate reader (MPR-Ai, TOSOH, Tokyo, Japan) at 570 nm. IL-2 activity was expressed in relative activity as stimulation index (SI) as described previously (Xu et al. 1993).

All data in the present study were expressed as mean value ± standard deviation. Two-way analysis of variance (ANOVA) was used for statistical analysis without stated. $P<0.05$ was considered to be significant difference.

RESULTS AND DISCUSSION

In order to examine the cross-sensitivity between DNBS and DNCB in vivo, mouse ear swelling test was employed in this study. As shown in Table 1, the marked mouse ear swelling responses were elicited by topical

application of 1% DNBS both in 0.5 and 2.5% DNCB-sensitized mice. And, the mouse ear response observed in 2.5% DNCB-sensitized mice was significantly different from that in mice sensitized with 0.5% DNCB. These data confirmed the cross-sensitivity between DNBS and DNCB in vivo. Further, we tested this cross-sensitivity using hapten-induced in vitro IL-2 production as an index. Table 2 shows that DNBS at three concentrations caused significant in vitro IL-2 release by the lymph node cells from both 0.5 and 2.5% DNCB-sensitized mice. And the dose-responses for DNBS-induced in vitro IL-2 production by the lymph node cells were demonstrated in both sensitized groups. The spearman's coefficients for control, 0.5% and 2.5% DNCB-sensitized groups were -0.031, 0.625 ($p<0.01$) and 0.792 ($p<0.01$), respectively. Moreover, DNBS-induced in vitro IL-2 production by the lymph node cells from 2.5% DNCB-painted mice were significantly higher than those in 0.5% DNCB-painted murine lymph node cells, suggesting that DNBS-induced in vitro IL-2 production was also dependent on DNCB dose used for in vivo sensitization. Furthermore, to examine if DNBS-induced in vitro IL-2 production by the DNCB-sensitized murine lymph node cells reflects the cross-sensitivity of DNBS with DNCB in vivo the correlation between DNBS-elicited ear swelling response and DNBS-induced in vitro IL-2 secretion was calculated. A good positive correlation between DNBS-induced mouse ear swelling response and 100 ppm DNBS-stimulated in vitro IL-2 release by the lymph node cells was observed (Fig.1). The similar results were obtained when the lymph node cells from DNCB-primed mice was stimulated with 50 ppm or 25 ppm DNBS in vitro. The correlation coefficients for 100 ppm, 50 ppm and 25 ppm DNBS were 0.765, 0.842 and 0.685, respectively.

It has been documented that contact hypersensitivity is generally mediated by type-1 helper T cells (Th1 cells) (Mossmann and Coffman 1989). Th1 cells secrete IL-2, IL-3, IFN-gamma, tumor necrosis factor-alpha and macrophage-granulocyte colony stimulating factor. Among these cytokines, IL-2 and IFN-gamma are the key cytokines in the development of contact hypersensitivity (Kelley et al. 1986; Zaloom et al. 1991; Wei et al. 1993). Our previous study (Xu et al. 1993) and other studies (Nordlind and Liden 1993, Koga et al. 1993) have reported that contact allergen-induced in vitro IL-2 and IFN-gamma productions by murine lymph node cells or human peripheral blood lymphocytes are valuable tools for identifying contact sensitizers in vitro. This study was designed to further address whether this method can be applied to test cross-sensitizing potentials of chemicals.

Although the cross-sensitivity between DNCB and DNBS has previously been demonstrated by various experimental systems including guinea pig maximization test and in vitro lymphocyte proliferation test (Li and Aoyama

Table 1. Mouse ear swelling responses induced by 1% DNBS in DNCB-sensitized and control mice

DNCB sensitizing dose	Ear challenged by solvent	Ear challenged by 1% DNBS
	Percent increase in ear thickness	
Control	1.6 ± 2.2	2.9 ± 3.1
0.5% DNCB	2.2 ± 2.3	8.0 ± 3.4[a,b]
2.5% DNCB	2.4 ± 1.9	34.4 ± 9.6[a,c]

[a]$p<0.01$ compared with the ear challenged by solvent in the same group.
[b]$p<0.05$ compared with the ear challenged by 1% DNBS in the control group.
[c]$p<0.01$ compared with the era challenged by 1% DNBS in 0.5% DNCB group.

Table 2. DNBS-stimulated in vitro IL-2 production by the lymph node cells from control and DNCB-sensitized mice

DNCB sensitizing dose	DNCB concentration used for in vitro stimulation		
	25 ppm	50 ppm	100 ppm
Control	2.1 ± 0.9	2.3 ± 1.2	1.9 ± 0.9
0.5% DNCB	8.6 ± 2.4[a]	9.9 ± 1.6[a]	12.9 ± 3.3[b]
2.5% DNCB	16.2 ± 3.2[b,c]	23.1 ± 4.4[b,d]	23.7 ± 3.7[b,c]

[a]$p<0.05$ and [b]$p<0.01$ compared with the control group.
[c]$p<0.05$ and [d]$p<0.01$ compared with 0.5% DNCB group.

1992; Garrigue et al. 1994), our study is the first approach to assess their cross-sensitivity using hapten-stimulated in vitro IL-2 production by murine lymph node cells. Using this model, we confirmed that DNBS-stimulated in vitro IL-2 production by the lymph node cells from DNCB-sensitized mice displayed a dose-dependent fashion with respect to in vivo DNCB sensitizing dose and in vitro DNBS-stimulating concentration. Moreover, DNBS-induced in vitro IL-2 production by the DNCB-sensitized murine lymph node cells was positively correlated with DNBS-elicited mouse ear swelling response. In agreement with our findings, the role of IL-2 in contact hypersensitivity is supported by a large body of

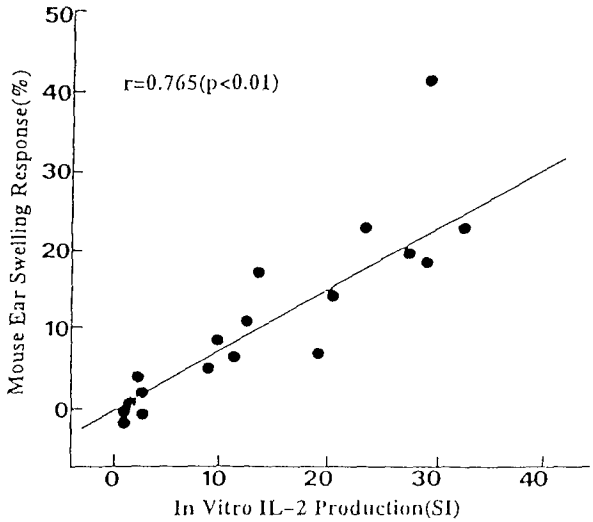

Figure 1. Correlation between 1% DNBS-elicited mouse ear swelling response and 100 ppm DNBS-induced in vitro IL-2 production by the lymph node cells from mice skin-painted by acetone/olive oil, 0.5 and 2.5% DNCB.

evidence. For example, Kelley et al (1986) reported that anti-IL-2 receptor antibody depressed the in vivo hypersensitivity through elimination of IL-2 receptor-bearing cells, whereas Zaloom et al (1991) observed that in mice delayed type hypersensitivity to DNCB was enhanced following in vivo administration of IL-2 at the eliciting phase. Mohler & Butler (1990) detected an increased IL-2 mRNA 3 days after contact sensitization with picryl chloride. More recently, Tsicopoulos et al (1992) reported that the cells from tuberculin biopsies, a classical delayed type hypersensitivity in humans, expressed IL-2 and IFN-gamma mRNAs preferentially. Therefore, we assume that DNBS-induced in vitro IL-2 production may reflect the cross-sensitivity of DNBS with DNCB in vivo and this may be used for evaluating cross-sensitivity among the chemicals.

In this study, because DNBS-stimulated in vitro lymphocyte proliferation was not performed we can not directly compared the results between hapten-induced in vitro IL-2 production and lymphocyte proliferation test. However, it seems that the present model is different from hapten-stimulated in vitro lymphocyte proliferation test at three points. First, the fundamentals of our model focuses on determination of hapten-stimulated in vitro IL-2 production, one of key cytokines exclusively produced by Th1 cells responsible for delayed-type hypersensitivity. So, it may be superior to crude hapten-stimulated lymphocyte proliferation, which determines a consequence of lymphocytes in response to the various cytokines including

IL-2. In this case, other coexisting cytokines may synergies or antagonize IL-2-driven lymphocyte proliferative response which in turn probably overestimates or underestimates the results. Second, although the basis for the lymphocyte proliferation test is that circulating memory T cells will proliferate in response to a specific antigen, some metals have been reported to stimulate significant no-specific lymphocyte proliferation (Nordlind 1984 & 1985). As regarding hapten-stimulated cytokine production, we think that it may be useful for distinguishing specific reaction from no-specific reaction. As evidence, Koga et al (1993a,b) reported that gold and nickel can not only significantly stimulate lymphocyte proliferation, but also induce IL-2 and interferon-gamma productions by peripheral blood mononuclear cells from patients with gold or nickel contact allergy. Finally, it should be noted that at present our model can not be simply extended to guinea pigs this is largely because of our poor understandings of immunology in guinea pigs and no suitable methods available for determination of guinea pig IL-2.

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