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Contact allergic response to dinitrochlorobenzene (DNCB) in rats: Insight from sensitization phase

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ABSTRACT

Contact hypersensitivity (CHS) is a T-cell-mediated skin inflammatory reaction to cutaneous exposure to small sensitizing chemicals, haptens. Majority of CHS studies were conducted in mice and there is paucity of data in other experimental animals. In the present study, characteristics of contact hypersensitivity reaction to dinitrochlorobenzene (DNCB) were determined in Th1-prone Dark Agouti (DA) rats by evaluating sensitization phase as a function of time-dependent changes in draining lymph nodes (DLN). Apart from basic indices of DLN activity (cellularity and proliferation), the production of cytokines relevant for CHS induction, interleukin-6 (IL-6), interferon- γ (IFN- γ), interleukin-17 (IL-17) and interleukin-4 (IL-4) was analyzed. Anti-inflammatory cytokine interleukin-10 (IL-10) production by DLN cells was determined as well. Highest production of IL-6, IFN- γ and IL-17 in sensitized animals was observed at day 3 after DNCB application, with a decrease at day 5. Increased messages for IFN- γ and IL-17 were noted at this time point. In contrast to inflammatory cytokines, anti-inflammatory cytokine interleukin-4 (IL-4) was undetectable during the entire sensitization phase. Differential pattern (IL-6 and IFN- γ) and level (IFN- γ and IL-17) of inflammatory cytokine production was noted in sensitized Th2-prone Albino Oxford (AO) rats. Similarly to DA rats, no changes in IL-4 were noted in AO rats. Strain-dependent differences in inflammatory cytokine production seem to be based on anti-inflammatory cytokine interleukin-10 (IL-10). Production of IFN- γ concomitantly with undetectable IL-4 in both strains classify rat CHS to DNCB as Th1/type 1 reaction. Detection of IL-17 in sensitized DLN cells points to the involvement of T_H17 cells in rat contact hypersensitivity.

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Introduction

Contact hypersensitivity (CHS) is a T-cell-mediated skin inflammatory reaction to cutaneous exposure to small sensitizing chemicals, haptens. As shown in murine models of contact hyper-

sensitivity to strong haptens such as dinitrohalobenzenes and oxazolone (OXAZ), two distinct phases are necessary to produce the full CHS reaction. The sensitization phase (afferent or induction phase) starts with the first contact of skin with the hapten, after its topical application to animal's abdominal or dorsal area. Hapten-loaded skin-derived dendritic cells that migrate to the draining lymph nodes present haptenated peptides to hapten-specific CD4⁺ and CD8⁺ T cells, stimulating their activation and proliferation (Bour et al. 1995). Numerous cytokines are produced during the sensitization phase in murine CHS to haptens, including IL-1, IL-2, IL-4, IL-6 and IFN- γ (Thomson et al. 1993; Hope et al. 1994a) and are thought to be involved in the development of CHS response. IL-1, IL-2 and IL-6 are required for the proliferation of draining lymph node cells, though some doubt has been cast upon the role of IL-6 in DLN cell proliferation (Lappin et al. 1998). Production of IFN- γ by draining lymph node cells following sensitization is considered important for the development of effector cells in CHS to dinitrofluorobenzene (DNFB) (He et al. 2006). Recently, production of IL-17,

Abbreviations: CHS, contact hypersensitivity; DNCB, dinitrochlorobenzene; DNFB, dinitrofluorobenzene; HMDI, dicyclohexylmethane-4,4'-diisocyanate; OXAZ, oxazolone; DA, Dark Agouti; AO, Albino Oxford; Le, Lewis; BN, Brown Norway; LLNA, local lymph node assay; DLN, draining lymph node; Th, T helper; Tc, T cytotoxic; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IFN- γ , interferon- γ ; IL, interleukin; mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction.

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major cytokine of Th17 cells, was shown during sensitization phase in mice (He et al. 2006) and its role in the generation of effector cells was suggested (He et al. 2009). The involvement of IL-4 in the induction of CHS to oxazolone and DNCB was noted in mice (Salerno et al. 1995; Traidl et al. 1999; Nagai et al. 2000; Yokozeki et al. 2000), but the exact role of IL-4 in the development of CHS is not yet known.

The elicitation phase (efferent or challenge phase) occurs following the application of hapten to the skin, usually on the ears, of sensitized animals several days later. Hapten diffusion is associated with pro-inflammatory innate immune cell activity in challenged skin (Dilulio et al. 1999; Zhang and Tinkle 2000), with the resulting infiltration of hapten-primed T cells in epidermis and dermis (Engeman et al. 2004). IFN- γ -producing CD8⁺ T cells are considered to be the primary cutaneous effector cells in mice (Gocinski and Tigelaar 1990; Xu et al. 1996) thus qualifying CHS as Th1/type1 T cell-mediated hypersensitivity reaction in the skin. However, the involvement of both CD8⁺ and CD4⁺ cells as effector IFN- γ producing T cells in CHS was shown in other studies (Wang et al. 2000; Saint-Mezard et al. 2004; He et al. 2009). Moreover, the importance of Th2 anti-inflammatory cytokine interleukin-4 (IL-4) in CHS expression was noted, as impairment of CHS was demonstrated in IL-4 deficient mice (Weigmann et al. 1997; Traidl et al. 1999). In contrast, it was shown that IL-4 producing CD4⁺ T cells negatively regulate CHS (Xu et al. 1996). Another Th2 anti-inflammatory cytokine, interleukin-10 (IL-10), is considered to be a natural regulator of CHS expression in mice (Ferguson et al. 1994), as IL-10 neutralization prolonged the duration of CHS, and injection of rIL-10 before challenge prevented the elicitation of CHS reaction. According to some, contact hypersensitivity responses should not be classified strictly as Th1 or Th2, as both Th1 and Th2 cells and their signature cytokines play a role in murine CHS (Ulrich et al. 2001; Watanabe et al. 2002).

From the above cited data it is clear that, although murine CHS is much studied, the knowledge of its mechanisms is still far from complete. In addition, there is paucity of data about CHS in other experimental animals, which might increase our understanding of the immunobiology of contact allergy. More than 20 years ago, rat CHS has been proposed as an experimental model for contact allergy studies (Scheynius et al. 1988), but a comparatively low amount of data has been gathered since. The involvement of IFN- γ in the effector phase of rat CHS was shown (Skoglund et al. 1988; Skoglund and Scheynius 1990) and a relevance of Th1 response for the expression of CHS was suggested based on a more intense ear skin response in Th1-prone Lewis (Le) rats as compared to Th-2 prone Brown Norway (BN) rats (Peszowski et al. 1994), as well as on Th1-promoting cytokine IL-12 production by DLN cells in the effluent phase of the reaction in BN rats (Dearman et al. 2002). Mechanisms of CHS induction in rats are virtually unexplored. Data on the increase in draining lymph node weight and numbers of dividing (BrdU⁺) cells following primary sensitization of ear skin with DNCB in the local lymph node assay (LLNA) provided some insight into the events that follow sensitization (Arts et al. 1996).

In the present study, CHS to dinitrochlorobenzene (DNCB), as a representative of nitrohalobenzenes, most often used group of haptens in studies of murine CHS, was explored by evaluating the characteristics of induction of the reaction, i.e. afferent/sensitization phase, the phase with which CHS begins and upon which it depends. Beside basic parameters of activity (cellularity and proliferation) of draining lymph nodes, the production of cytokines relevant for CHS induction, including IL-6, thought to be involved in non-specific aspects of CHS induction, as well as IFN- γ , IL-4 and IL-17, as major cytokines associated with Th cell subsets, were measured. The production of anti-inflammatory cytokine IL-10 was measured as well. DLN cell activity during the sensitization phase of CHS to DNCB was determined in inbred Dark Agouti

(DA) rats, with a propensity of developing Th1-skewed responses to inflammatory stimuli (Mijatović et al. 1997). For comparison, DLN cell cytokine production was determined in Th2-prone Albino Oxford (AO) rats as well. Presented data are the first to describe draining lymph node cytokine response during the afferent phase of CHS to DNCB in rats, as well as strain-dependent differences in cytokine profile during the induction of this reaction.

Materials and methods

Chemicals

One-chloro-2,4-dinitrobenzene (DNCB) is obtained from BDH Chemicals Ltd., UK and dissolved in 4:1 acetone:olive oil (vehicle). Two, four-dinitrobenzenesulfonic acid (DNBS) (Aldrich Chemical Company Inc., Milwaukee, USA) was dissolved in culture medium. RPMI-1640 culture medium (PAA laboratories, Austria) supplemented with 25 mM HEPES, 2 mM glutamine, 20 mg/ml gentamycin, 5×10^{-5} M 2-mercaptoethanol (2-ME) and 5% (v/v) heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Austria) was used in cell culture experiments. Radiolabeled [³H]-thymidine was purchased from GE Healthcare, UK. FITC-labeled mouse antibodies to rat CD4 (IgG2a) and rat CD8 (IgG1) were purchased from eBioscience Inc. (San Diego, CA, USA). FITC-labeled mAb to TNP-keyhole limpet hemocyanin (mouse IgG2a, kappa) was purchased from BD Pharmingen.

Animals

Twelve weeks old male Dark Agouti (DA) and Albino Oxford (AO) rats, bred and conventionally housed at the Institute for Biological Research “Siniša Stanković”, Belgrade, Serbia, were used. They were maintained at 12 h photoperiod, 21/24 °C temperature control and water available *ad libitum*. Four–six animals were assigned to each treatment group per experiment, with at least three independent experiments. After treatment, animals were caged individually. Animal treatment has been carried out in adherence with the Ethical Committee of our Institute.

Contact sensitization with DNCB and ear swelling response

Animals received 100 μ l of 0.4% (w/v) DNCB in acetone/olive oil (vehicle) for 2 consecutive days (day 0 and day +1) at the upper part of the dorsum (approximately 16 cm²). On day +4 or +6 (i.e. three and five days following the last application of DNCB), rats were challenged by an application of 50 μ l of three times lower dose of DNCB (i.e. 0.13%) to the ventral and dorsal surfaces of the left ear (sensitization/elicitation regime 0.4%/0.13%). Control rats received vehicle (acetone/olive oil) at the dorsal area and the ears (unsensitized rats; sensitization/elicitation DNCB doses 0%/0%). One group of unsensitized rats was challenged with 0.13% DNCB (sensitization/elicitation DNCB doses 0%/0.13%). The intensity of CHS response was evaluated 24 h later by measuring ear swelling, which is an *in vivo* measure of contact hypersensitivity (CHS) response. Ear thickness was assessed blindly by a hand-held engineer's micrometer (at least six measurements per ear). An increase in ear swelling was defined as the difference between ear thickness 24 h after challenge and ear thickness before challenge.

Histology

Ear skin samples were taken 24 h following challenge and were fixed in 4% buffered formaldehyde (pH 6.9), embedded in paraffin wax for sectioning at 5 μ m and stained with hematoxylin/eosin (H&E).

Draining lymph node (DLN) cell proliferation

Lymph nodes that drain the areas where DNCB was applied for sensitization (scapular and axillary nodes) were harvested from both sensitized and non-sensitized animals at days 1, 3 and 5 following the last application of DNCB or vehicle, respectively. Cell suspensions of pooled (two scapular and two axillary per animal) draining lymph nodes were prepared by mechanical disintegration and cell numbers determined by using an improved Neubauer hemocytometer. Cell viability was determined by Trypan blue dye exclusion and was more than 95%. Pooled DLN cells were used for estimation of DLN cell activity. For draining lymph node cell proliferation measurement, cells were cultured at 0.5×10^6 /well in 96-well microplates (Flow, USA) for 16 h with $0.5 \mu\text{Ci}$ of [^3H]-thymidine per well added at the onset of culture. Incorporation of [^3H]-thymidine into cellular DNA was measured by liquid scintillation counting (LKB, Wallac). Proliferation is expressed as counts per minute (c.p.m.).

Flow cytometry

Draining lymph node cells isolated at days 3 and 5 following the last application of DNCB or vehicle were incubated on ice in a volume of $100 \mu\text{l}$ with FITC-conjugated mouse mAbs to rat CD4 or rat CD8. FITC-labeled irrelevant mAb to TNP-keyhole limpet hemocyanin was used as isotypic control. After washing, the cells were fixed and assayed for fluorescence intensity on a FACScan cytometer (Becton Dickinson Immunocytometry Systems, USA).

Draining lymph node (DLN) cell cytokine production

DLN cells isolated at days 1, 3 and 5 following the last application of DNCB or vehicle were cultured at 5×10^6 /ml in 24-well plates for 72 h in medium solely (spontaneous *ex vivo* cytokine production) or in the presence of DNBS, water-soluble analogue of DNCB (DNBS-stimulated production). Cytokine production was determined in the conditioned medium using commercially available ELISA sets for rat IL-6 (R&D Systems, Minneapolis, USA), rat IFN- γ (BD Biosciences, San Diego, CA, USA), rat IL-4 (BD Biosciences, San Diego, CA, USA), rat IL-10 (BD Biosciences, San Diego, CA, USA) and mouse IL-17, cross-reactive with rat IL-17 (eBioscience Inc., San Diego, CA, USA). Tests were performed according to the manufacturer's recommendations. Cytokine titer was calculated using a standard curve constructed by known amounts of respective recombinant cytokines.

Real-time polymerase chain reaction (RT-PCR)

In order to determine cytokine gene expression in DLN cells, real-time PCR was performed. Draining lymph node cells isolated at day 3 following the last DNCB application, i.e. at the time point of maximal cell cytokine production, were used. First, total RNA was isolated from the cells using mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) and reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase, according to manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified using Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the recommendations of the manufacturer in the total volume of $20 \mu\text{l}$ in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermocycler conditions comprised the initial step at 50°C for 5 min, followed by a step at 95°C for 10 min and subsequent 2-step PCR program at 95°C for 15 s and 60°C for 60 s for 40 cycles. The PCR primers (Metabion) were as follows: IFN- γ forward primer 5'-TGG CAT AGA TGT GGA AGA AAA GAG-3'; IFN- γ reverse primer 5'-TGC AGG ATT TTC ATG TCA CCA T-3'; IL-6 for-

ward primer 5'-GCC CTT CAG GAA CAG CTA TGA-3'; IL-6 reverse primer 5'-TGT CAA CAA CAT CAG TCC CAA G-3'; IL-17 forward primer 5'-ATC AGG ACG CGC AAA CAT G-3'; IL-17 reverse primer 5'-TGA TCG CTG CTG CCT TCA C-3'; β -actin forward primer 5'-GCT TCT TTG CAG CTC CTT CGT-3'; β -actin reverse primer 5'-CCA GCG CAG CGA TAT CG-3'. Accumulation of PCR products was detected in real time and the results were analyzed with 7500 System Software (AB) and presented as 2^{-dC_t} , where dC_t is the difference between C_t values of the specific gene and that of endogenous control (β -actin).

Statistics

Results were pooled from at least three experiments and expressed as mean values $\pm$ SD. Mann-Whitney *U* test was used for the analysis of functional studies/cell culture data, and Student's *t*-test for gene expression. Correlations were analyzed by linear regression. *P* values < than 0.05 were considered significant.

Results

Ear swelling response

Measurements of ear thickness following challenge of unsensitized (DNCB 0%) or previously sensitized (DNCB 0.4%) rats and skin histology are presented in Fig. 1. No change in ear thickness was noted following challenge of non-sensitized animals with either 0% or 0.13% DNCB. Challenge of sensitized animals with 0.13% DNCB resulted in a significant increase of ear thickness, as compared to non-sensitized animals (Fig. 1A). Similar intensity of response was noted when challenge was conducted on days 3 and 5 after the last DNCB application. Histology of ear skin in animals challenged three days following sensitization revealed inflammation with characteristic tissue edema and leukocyte infiltration (Fig. 1D), as compared to ear skin of unsensitized rats challenged with 0.13% DNCB (Fig. 1C) or with vehicle solely (Fig. 1B). A similar histological picture was noted when challenge was conducted five days following the last application of DNCB.

Draining lymph node cell numbers, proliferation and T cell subset composition during the sensitization phase

As shown in Fig. 2A, an increase in numbers of cells in draining lymph nodes of sensitized animals, as compared to non-sensitized animals, was observed following DNCB application. Rise in DLN cellularity was noted as early as day 1 following sensitization, followed by days 3 and 5, when highest numbers of cells were noted ($p < 0.01$ vs. day 1). No differences in DLN cell numbers were detected between day 3 and day 5 after DNCB application. Determination of proliferative capacity of DLN cells from sensitized rats (Fig. 2B) showed increased proliferation of DLN cells from sensitized, as compared to non-sensitized animals, starting from day 1, with highest levels of proliferation at day 3 following DNCB application ($p < 0.001$ compared to day 1 and $p < 0.01$ compared to day 5). Significant correlation was noted between proliferation level and cell numbers during sensitization phase ($y = 13.49 + 0.03 \times X$; $r = 0.89$, $p < 0.001$).

Phenotypic analysis of DLN T cell subset composition performed at days 3 and 5 following DNCB application revealed no changes in relative numbers of CD4⁺ and CD8⁺ T cells in sensitized, as compared to non-sensitized animals at both time points (Table 1). Due to an increase in DLN cellularity at these time points, higher numbers of both CD4⁺ and CD8⁺ T cells were found in sensitized animals.

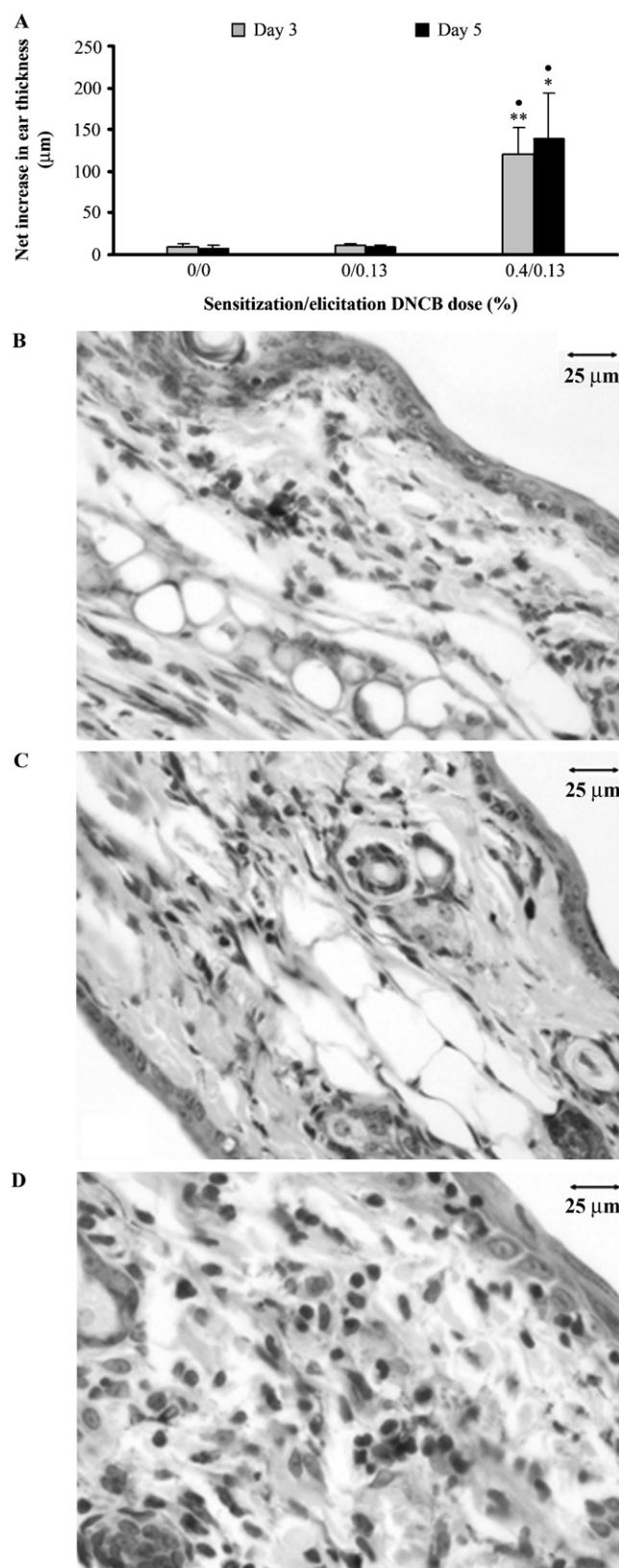


Fig. 1. Basic characteristics of ear swelling response to challenge with DNCB in sensitized rats. Mean increase in ear thickness (A). Histology of ear skin of non-sensitized rats challenged with vehicle solely (B) or 0.13% DNCB (C) or sensitized rats challenged with 0.13% DNCB (D). Results were pooled from at least three experiments with 4–6 animals per group and expressed as mean values $\pm$ SD. Significance at * $p < 0.05$ and ** $p < 0.01$ vs. non-sensitized rats (0%/0% DNCB) and * $p < 0.05$ vs. non-sensitized rats challenged with 0.13% DNCB.

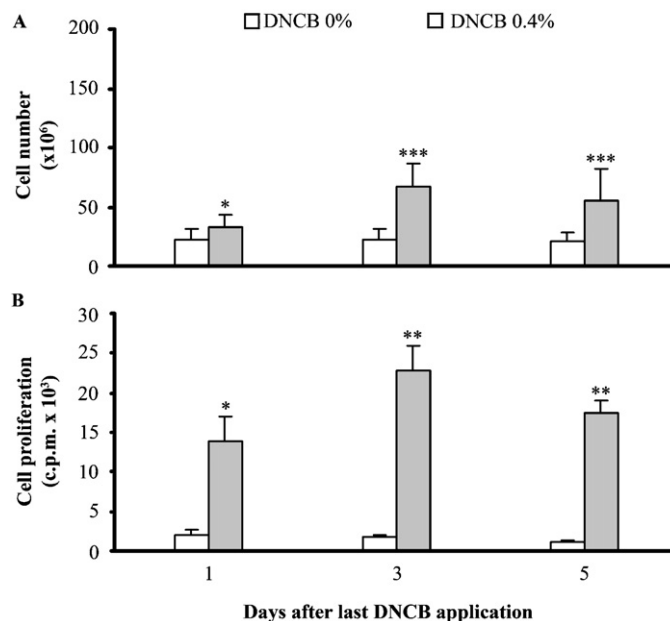


Fig. 2. Cellularity and proliferation of draining lymph node cells during sensitization phase. Draining lymph node cell numbers (A) and proliferation (B). Results were pooled from at least three experiments with 4–6 animals per group and expressed as mean values $\pm$ SD. Significance at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. non-sensitized (0%/0% DNCB) rats.

Draining lymph node cell interleukin-6 (IL-6) production during sensitization phase

Determination of the dynamics of production of IL-6 during the sensitization phase (Fig. 3) revealed high spontaneous draining lymph node cell IL-6 production in both sensitized and non-sensitized rats at day 1 following sensitization or vehicle application, respectively (Fig. 3A). However, owing to high levels of IL-6 produced by DLN cells of non-sensitized animals, IL-6 was only slightly ($12 \pm 9\%$), but still significantly ($p < 0.05$), higher in sensitized, as compared to non-sensitized animals. Three days following DNCB or vehicle application, IL-6 production by DLN from non-sensitized animals decreased sharply, while more than 300% ($347 \pm 139\%$) higher IL-6 production at this time point was noted in sensitized, compared to non-sensitized animals. Five days following sensitization IL-6 was not detectable. DNBS stimulation resulted in similar levels and pattern of IL-6 production during the afferent phase (Fig. 3B). Determination of mRNA for IL-6 conducted at day 3 (when increase in *ex vivo* production of IL-6 by sensitized DLN cells was noted, with relatively low production of this cytokine by DLN cells from non-sensitized animals) revealed no changes in IL-6 mRNA levels in DLN from sensitized, as compared to non-sensitized animals (Fig. 3C).

Draining lymph node cell IFN- γ , IL-17 and IL-4 production during sensitization phase

Sensitization with DNCB resulted in increase in DLN cell production of pro-inflammatory cytokines IFN- γ and IL-17 during the sensitization phase (Fig. 4). Increased spontaneous production of IFN- γ was noted at all time points examined, with the highest levels at day 3 following the last DNCB application (Fig. 4, panel A). Similar pattern of IFN- γ production was noted following stimulation with DNBS *in vitro*, with higher levels ($p < 0.01$) at days 3 and 5 compared to spontaneous production at these time points (Fig. 4, panel A). Determination of mRNA for IFN- γ performed at day 3, when most intense production of this cytokine was observed,

Table 1Draining lymph node CD4⁺ and CD8⁺ cell subset composition during sensitization phase.

Days after last DNCB application	DNCB dose (%)	CD 4 ⁺		CD 8 ⁺		CD4 ⁺ /CD8 ⁺
		Relative cell number (%)	Total cell number (×10 ⁶)	Relative cell number (%)	Total cell number (×10 ⁶)	
Three	0	56.9 ± 3.3	12.8 ± 4.8	20.5 ± 1.0	4.6 ± 1.7	2.8 ± 0.3
	0.4	56.1 ± 0.01	40.5 ± 12.3***	18.9 ± 3.2	13.6 ± 4.2***	3.0 ± 7.7
Five	0	60.4 ± 1.1	12.7 ± 7.1	25.2 ± 1.8	5.3 ± 2.9	2.4 ± 0.2
	0.4	58.3 ± 3.3	35.0 ± 17.2***	22.6 ± 1.8	13.6 ± 6.7**	2.6 ± 0.1

Significantly different at ** $p < 0.01$, *** $p < 0.001$ from non-sensitized (0%/0% DNCB) rats.

showed a significant increase in DLN cell IFN- γ mRNA, as compared to non-sensitized animals (Fig. 4, panel A).

Increase of spontaneous IL-17 production was noted at day 3 following DNCB application (Fig. 4, panel B). DNBS stimulation resulted in slight, but significantly ($p < 0.05$ vs. spontaneous production) higher IL-17 content in the conditioned medium of DLN cells from sensitized rats at this time point, as well as at day 5 (Fig. 4, panel B). Determination of mRNA for IL-17 at day 3 following DNCB application showed a significant increase in DLN cell IL-17 mRNA, as compared to non-sensitized animals (Fig. 4, panel B).

In contrast to pro-inflammatory cytokines, both spontaneous and DNBS-stimulated IL-4 levels were undetectable (below the assay limits of 1.6 pg/ml) in DLN cell cultures.

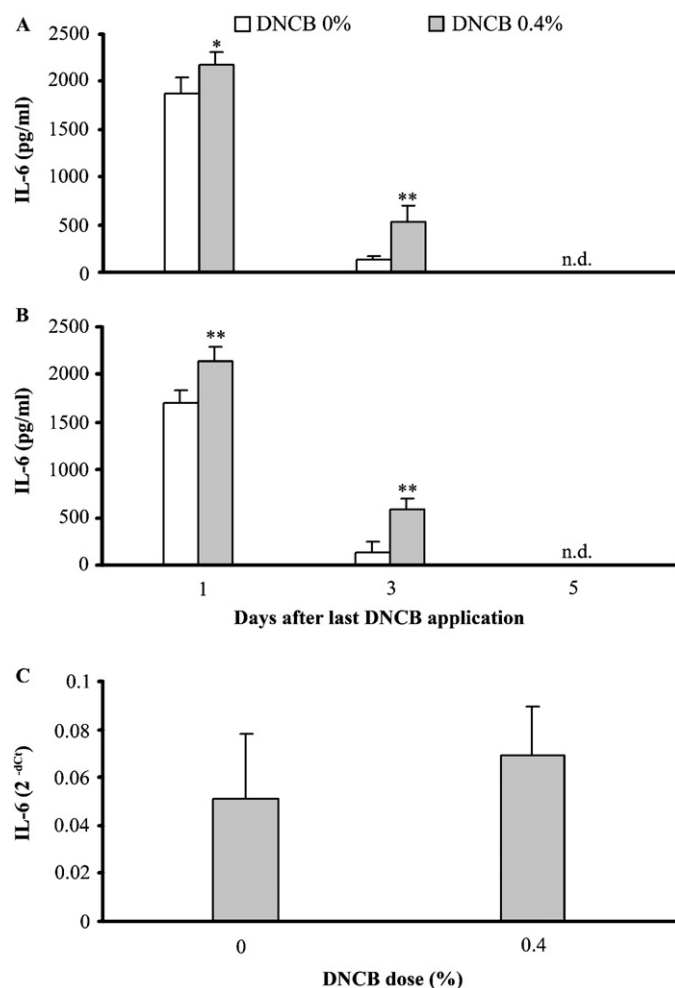


Fig. 3. Interleukin-6 (IL-6) production by draining lymph node cells during sensitization phase. Spontaneous production (A), DNBS-stimulated production (B), cytokine gene expression in draining lymph node cells three days following the last DNCB application (C). Results were pooled from at least three experiments with 4–6 animals per group and expressed as mean values ± SD. Significance at * $p < 0.05$, and ** $p < 0.01$ vs. non-sensitized (0%/0% DNCB) rats.

Draining lymph node cell IL-6, IFN- γ , IL-17 and IL-4 production in AO rats

The above presented data showed that sensitization of DA rats stimulated inflammatory cytokine production with unchanged anti-inflammatory IL-4 production. To obtain information whether such sensitization-induced cytokine profile is strain-dependent, the production of these cytokines during sensitization phase was measured in Th2-prone AO rats and compared to the pattern and the level of production of the same cytokines in DA rats (Fig. 5). Unchanged and slightly, but significantly lower spontaneous DLN cell IL-6 production was noted at days 1 and 3 following DNCB application, respectively, in sensitized as compared to non-sensitized AO rats; in contrast to this, in DA rats, an increase was observed at both time points (Fig. 5, panel A). Similar data were obtained following DNBS stimulation (Fig. 5, panel B). Increase in both spontaneous and DNBS-stimulated IFN- γ production was noted at day 3 and 5 following DNCB application in AO rats, in contrast to DA rats, where production of this cytokine in sensitized animals was detectable earlier (day 1) and was lower compared to DA rats. Similar to DA rats, increased spontaneous, as well as DNBS-stimulated IL-17 production was observed at day 3, but was significantly lower compared to DA rats. IL-4 production was undetectable during the sensitization phase in AO rats.

Draining lymph node cell interleukin-10 (IL-10) production by DA and AO rats

In the light of data that showed lower levels of produced IL-6, IFN- γ and IL-17 in AO rats, production of IL-10, anti-inflammatory cytokine known to inhibit the effects of these inflammatory cytokines in various experimental systems, was examined next. As shown in Fig. 6, a significantly higher IL-10 production was noted in sensitized animals of both strains three days following DNCB application, compared to non-sensitized individuals of the respective strain; however, the increase was significantly higher in AO (116% ± 39%) as compared to DA (36% ± 6%) rats.

Discussion

The present study was carried out in order to investigate the mechanisms of contact hypersensitivity to DNCB in rats by evaluating characteristics of the afferent phase of reaction (sensitization), the phase with which CHS begins and upon which it depends. In line with data from local lymph node assays in mice (Hope et al. 1994b) and rats (Arts et al. 1996), an increase in DLN cell numbers and *ex vivo* proliferation, the immunological correlates of hapten's contact-sensitizing potential, was noted in rats sensitized with DNCB. Higher DLN cellularity in sensitized, as compared to non-sensitized rats might be a consequence of cell migration to lymph nodes and/or cell proliferation. Highly significant correlation between DLN cell numbers and proliferation following DNCB application implies a significant contribution of proliferative activity to DLN cellularity during the sensitization phase. Both CD4⁺ and CD8⁺

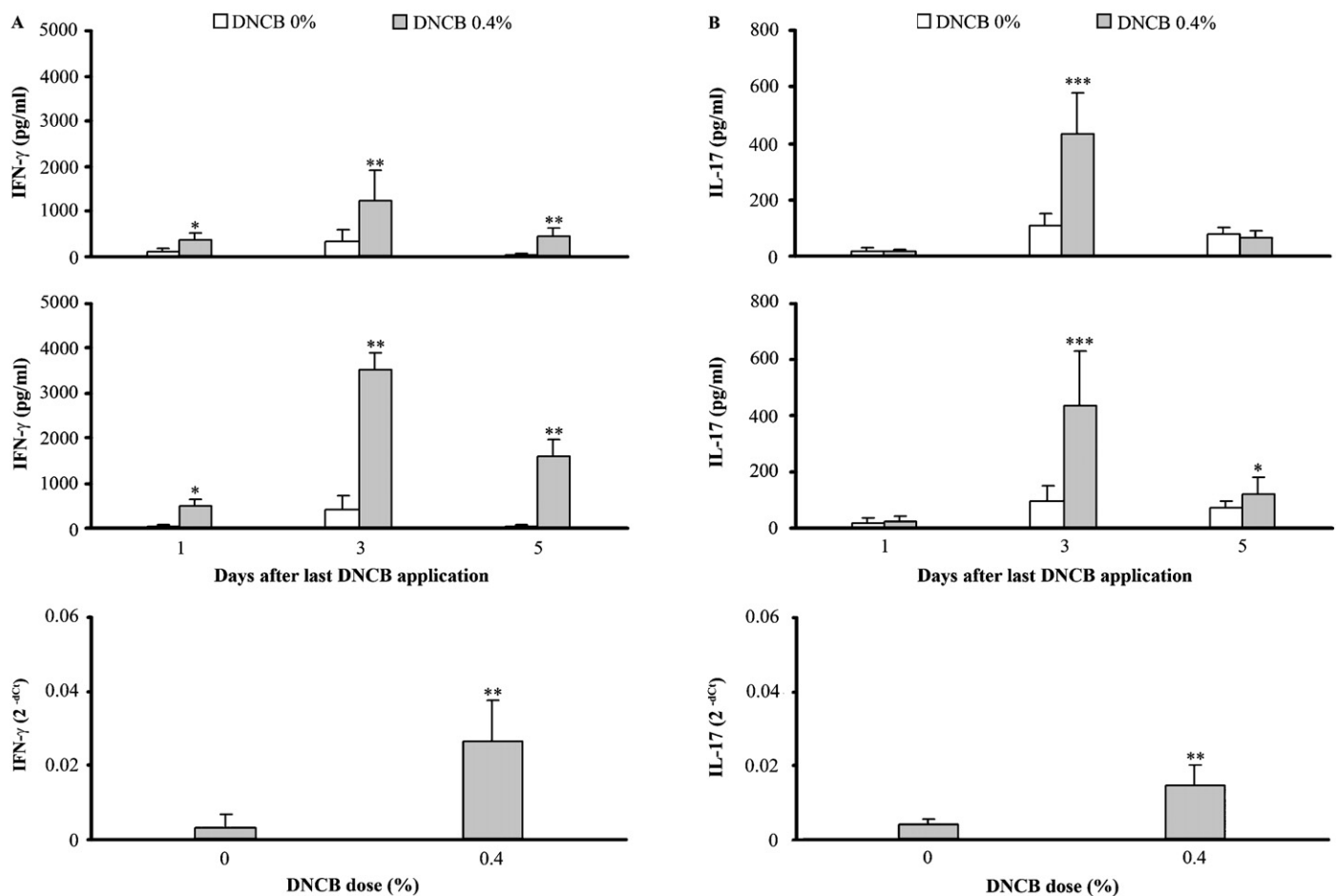


Fig. 4. Interferon- γ (IFN- γ) and interleukin-17 (IL-17) production by draining lymph node cells during sensitization phase. (Panel A) Spontaneous IFN- γ production (above); DNBS-stimulated IFN- γ production (middle); Gene expression three days following the last DNCB application (below). (Panel B) Spontaneous IL-17 production (above); DNBS-stimulated IL-17 production (middle); gene expression three days following the last DNCB application (below). Results were pooled from at least three experiments with 4–6 animals per group and expressed as mean values $\pm$ SD. Significantly different at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. non-sensitized rats (0%/0% DNCB).

cell proliferation and/or migration to DLN might have accounted for the observed increase in cellularity, as similar ratio of these subsets was noted during sensitization.

Increase in production of IL-6 by draining lymph node cells of sensitized animals is in line with data from murine CHS to other types of haptens such as dicyclohexylmethane-4,4'-diisocyanate (HMDI) and oxazolone (OXAZ) (Hope et al. 1994a,b) and shows, for the first time, that sensitization of rats with DNCB results in the production of this cytokine as well. Increase of IL-6 production by DLN cells from sensitized DA rats at day 3 post-sensitization (when no changes in IL-6 mRNA of DLN cells was noted) might be attributed to the contribution of various cell-derived stimuli present during 72-h DLN cell culture. High production of IL-6 by DLN cells from non-sensitized rats might be explained by the contribution of vehicle (acetone/olive oil), as some plant oils have been shown to increase IL-6 production by draining lymph node cells (Hope et al. 2000). Lack of responsiveness to DNBS stimulation implies that IL-6 might be involved in non-specific aspects of CHS induction. In this regard, the role played by IL-6 in bystander DLN cell proliferation, which is not critical for the priming of hapten-specific cells, as well as the inducibility of DLN cell IL-6 production by the application of non-specific inflammatory stimuli with skin-irritating properties are shown (Hope et al. 2000). Lack of increase of IL-6 production at day 1 following DNCB application is in agreement with data that showed that sensitization may proceed in the absence of IL-6 in some strains of mice (Lappin et al. 1998).

Increase in spontaneous and DNBS-stimulated IFN- γ production, as well as IFN- γ gene expression, is in line with data on murine CHS to DNCB, as well as to similar haptens, such as DNFB (Ulrich et al. 2001; He et al. 2006). This confirms the importance of IFN- γ in contact hypersensitivity to the nitrohalobenzene class of haptens and shows that DNCB in rats, similarly to mice, provokes Th1/type 1 cytokine production and expression during the afferent phase of CHS. Both T cell subsets (along with NK cells, a rich source of IFN- γ) might have contributed to DLN cell cytokine production in rat CHS.

Increased production and expression of IL-17 by DLN cells of sensitized rats suggested further pro-inflammatory character of CHS to DNCB in rats and showed for the first time the involvement of this cytokine in the development of contact sensitivity in this animal species. Both CD4⁺ and CD8⁺ cells might be producers of IL-17 in rat CHS, as shown in murine CHS to DNFB (He et al. 2006, 2009).

A similar pattern of *ex vivo* and DNBS-stimulated production of IFN- γ and IL-17 *in vitro* indicates that the specificity of cytokine production during sensitization phase in rat hapten-primed production of IFN- γ and IL-17 by sensitized draining lymph node cells might be relevant for the development of effector cells. Hapten-primed IL-17-producing CD8⁺ T cells are necessary in the efferent phase of CHS to DNFB in mice (He et al. 2006). Along with IFN- γ -producing CD8⁺ T cells, CD8⁺ T cells that produce IL-17 initiate the immune response upon challenge with this hapten in sensitized mice (Kish et al. 2009). Both cytokines appear to be necessary for full CHS expression (He et al. 2009).

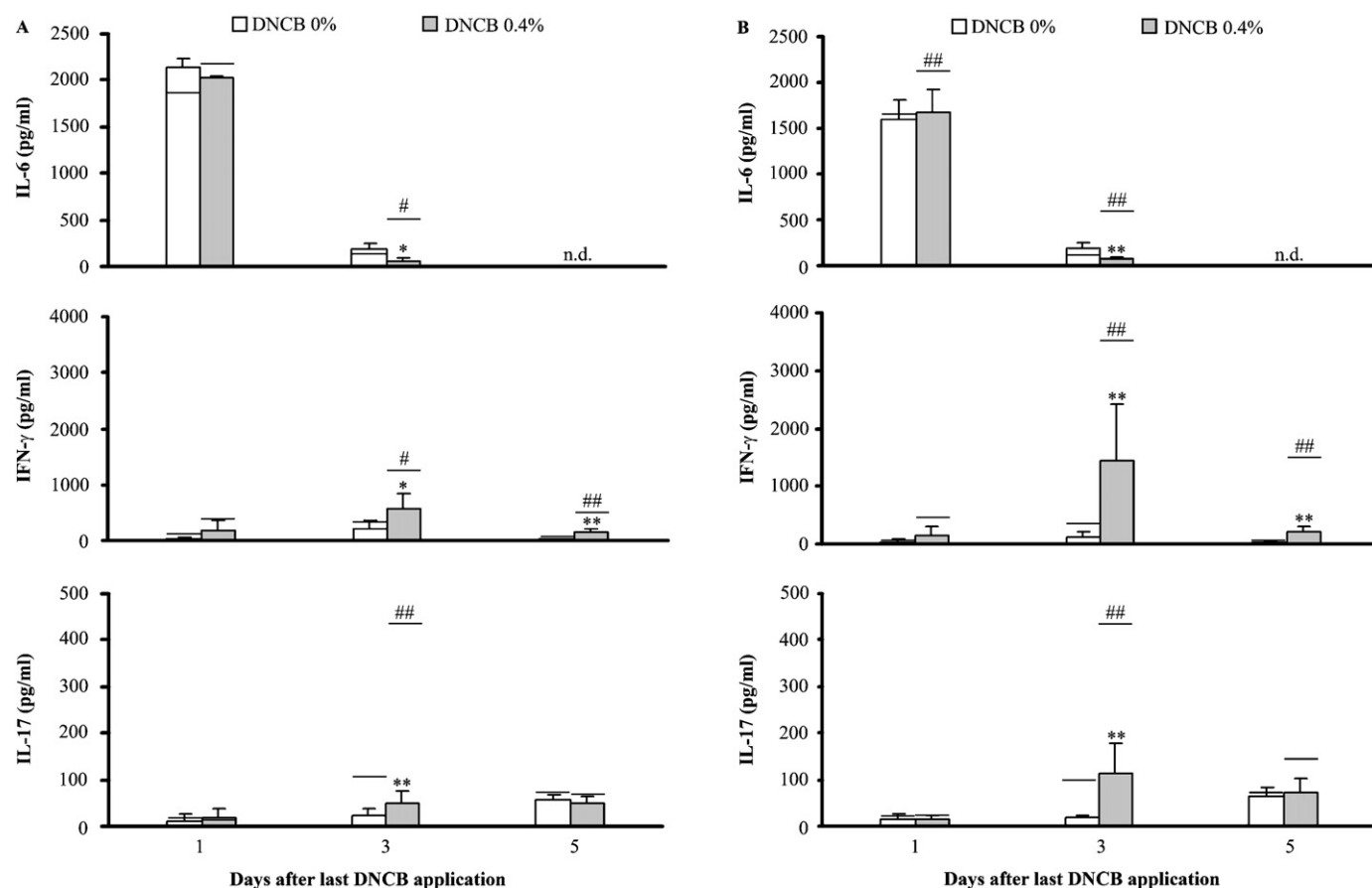


Fig. 5. IL-6, IFN- γ and IL-17 production by draining lymph node cells of AO rats during sensitization phase. (Panel A) Spontaneous IL-6 production (above); Spontaneous IFN- γ production (middle); Spontaneous IL-17 production (bellow). (Panel B) DNBS-stimulated IL-6 production (above); DNBS-stimulated IFN- γ production (middle); DNBS-stimulated IL-17 production (bellow). Results were pooled from at least three experiments with 4–6 animals per group. Columns represent mean values $\pm$ SD in AO rats. Bars represent mean values in DA rats. Significantly different at * $p < 0.05$ and ** $p < 0.01$ vs. non-sensitized AO rats (0%/0% DNCB). Significance at # $p < 0.05$ and ## $p < 0.01$ vs. AO rats.

Undetectable spontaneous, as well as DNBS-stimulated IL-4 production during the sensitization phase of CHS to DNCB in our study implies that this hapten did not provoke DLN cells to produce measurable amounts of this cytokine during CHS induction. This is in agreement with data that showed undetectable amounts of IL-4 produced in the efferent phase of CHS to DNCB in BN rats (Dearman et al. 2002) and BALB/c mice (Ulrich et al. 2001; Dearman et al. 2002), with detectable IL-4 obtained only following polyclonal stimulation of DLN cells with anti-CD3 antibodies or the lectin concanavalin A (ConA).

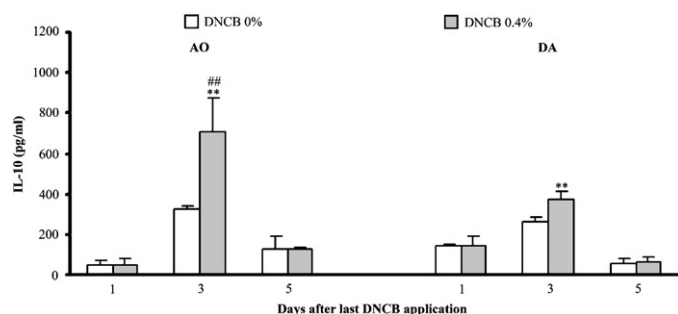


Fig. 6. Spontaneous interleukin-10 (IL-10) production by draining lymph node cells of AO and DA rats during sensitization phase. Results were pooled from at least three experiments with 4–6 animals per group and expressed as mean values $\pm$ SD. Significance at ** $p < 0.01$ vs. same-strain non-sensitized (0%/0% DNCB) rats; significance at ## $p < 0.01$ vs. DA rats.

Increase in IL-10 production during the sensitization phase of CHS to DNCB in rats is in line with data that showed an upregulation of IL-10 mRNA in DLN cells of DNFB-sensitized mice (Fujisawa et al. 1996). A decrease in IL-6 production noted at day 3, as well as lower levels of spontaneous IFN- γ and IL-17 DLN cell production in AO, as compared to DA rats might be ascribed to the action of IL-10, as this cytokine is a well known inhibitor of IL-6 production in a variety of experimental systems (Fiorentino et al. 1991); inhibition of Th1 responses (Trinchieri 2007) and of IL-17 production (Gu et al. 2008) by this cytokine has also been shown. Presence of IL-10 at day 3, when maximal DLN cell activity was noted, might reflect a host mechanism developed to avoid excessive T cell activation induced by haptens. The production of IL-10 by draining lymph node cells at day 3 following DNCB application observed in the present study reflects simultaneous activation of both Th2 (IL-10) and Th1/Tc1 (IFN- γ) producer cells, adding evidence that contact allergens elicit cytokine responses that do not necessarily strictly correspond to the classic Th1/Th2 concept (Ulrich et al. 2001). It should be noted, however, that with time following sensitization (i.e. at day 5), a drop in IL-10 production was seen, reaching levels similar to non-sensitized animals, while IFN- γ , although decreased compared to day 3, remained in sufficiently high concentration, confirming the predominant Th1/Type 1 character of CHS to DNCB in rats.

Drop in production of all cytokines examined at day five following DNCB application, along with a tendency of decrease in DLN cell proliferation, demonstrated a decline in regional lymph node immune response to sensitization with DNCB. This might be related to the intrinsic capacity of host immune system to regulate its response to chemicals, with likely involvement of regulatory

cells. Indeed, both CD4⁺ and CD8⁺ cells have been shown to regulate CHS responses in mice (Roncarolo et al. 2006; Cavani 2008).

In conclusion, based on the presented data, contact hypersensitivity to DNCB in rats shows similarity with murine CHS to dinitrohalobenzene haptens. As such, rat CHS is a useful model for studies of underlying immune mechanisms that might contribute to the understanding of this reaction in different species; this, in turn, might further be helpful in translational contact allergy studies. Characterization of experimental contact hypersensitivity in the rat, most often used animal in pharmacological/toxicological studies, might be useful in the elucidation of immunomodulatory potential of chemicals, particularly those that gain access to the host system via the skin, as well as in exploring the potential of therapeutic modalities to reduce skin damage caused by contact allergy.

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