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RESEARCH ARTICLE

Impact of the magnitude of sensitization dose on the incidence and intensity of CHS to dinitrochlorobenzene (DNCB): Insight from ear swelling and challenged-skin draining lymph node response in rats

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Abstract

Allergic contact dermatitis (ACD) is a common skin inflammatory disease that develops in hosts sensitized with contact allergens. Elucidation of dose–response relationships represents one of the approaches in studying the type of ACD in humans/animal models, termed as contact hyper-sensitivity reaction (CHS). Such studies have demonstrated that the intensity of sensitization determines the response to elicitation with a contact allergen, but underlying mechanisms are unclear. The aim of this study is to explore the impact of the sensitization on contact hypersensitivity to dinitrochlorobenzene (DNCB) in rats by measuring the incidence and intensity of a challenge response in hosts sensitized with two different doses (i.e. low and high) of this hapten. Assumptions concerning the contribution from the magnitude of sensitization doses were drawn on the basis of effects from the two doses on the measured reaction parameters. Ear swelling and activity of lymph nodes that drain challenged skin (cdLN), including cellularity, proliferation, and effector cytokine IFN γ and IL-17 production was measured in rats sensitized with 0.4% or 4% DNCB and challenged with a non-irritant (0.13%) dose. Sensitization with 4% DNCB resulted in a greater proportion of rats who responded more intensely (than unsensitized challenged rats) to challenge in terms of ear swelling and increases in cdLN activity (except for IFN γ). The intensity of cdLN responses was higher in these hosts as well. Among the high-dose-sensitized rats, greater cellularity/proliferation of cells from lymph nodes (sdLN) that drain the high-dose-sensitized skin, as well as higher IL-17 production, was noted compared to what was seen in rats that received low-dose sensitization. In contrast, unchanged spontaneous and even decreased hapten-stimulated IFN γ production after the high DNCB dose was observed. Based on the data, it seems the impact of magnitude of sensitization dose on CHS might be related to the rise in the proportion of rats that responded to challenge with an increase of dLN activity. Coincidental higher production of IL-17 by dLN cells from the high-dose-sensitized rats and following challenge of these hosts underscored the significance of IL-17 for a CHS to DNCB.

Keywords

Challenge response, contact hypersensitivity (CHS), dinitrochlorobenzene (DNCB), draining lymph nodes, interferon- γ (IFN γ), interleukin-17 (IL-17), rats

History

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Introduction

Allergic contact dermatitis (ACD) is a common skin inflammatory disease in humans, which develops at the site of skin re-exposure to contact allergen, in the individuals previously sensitized with same or a structurally closely related chemical (Saint-Mezard et al., 2004). The pathophysiology of ACD is studied in animals, mostly on mouse models referred to as contact hypersensitivity (CHS) reaction. It is a T-cell mediated inflammatory reaction, induced after epicutaneous application of small reactive chemicals, haptens. For full development of this reaction it is priming (sensitization) of hapten-specific T-cells that is necessary, with subsequent activation of hapten-specific effector

T-cells at the site of second hapten encounter, which mediates skin inflammation (elicitation phase). The sensitization (afferent) phase starts with the first skin contact with the hapten, after its topical application to the animal's abdominal or dorsal area. The elicitation (efferent or challenge) phase occurs following application of hapten to skin (usually on the ears of sensitized animals, several days later) and gives rise to a characteristic tissue edema and lesions (i.e. an ear swelling response) that reflect changes in cells in the affected skin (Grabbe and Schwarz, 1998) and draining lymph nodes (Hartmann et al., 2006).

One of the approaches in studying ACD in humans is elucidation of the dose–response relationships between the induction and elicitation phases of the reaction; these seem to determine the incidence as well as intensity of an ACD response (Friedmann and Moss, 1983; Friedmann, 2007). A quantitative relationship between the concentrations used for sensitization and elicitation of the allergic response to dinitrochlorobenzene (DNCB) was demonstrated both in humans (Friedmann and

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Moss, 1983; Friedmann, 2007) and mice (Scott et al., 2002). Exploration of the interaction between sensitization and challenge (elicitation) doses in human allergic response to DNCB demonstrated that the intensity of response (evaluated by an increase in skin-fold thickness, a traditional test which measures both sensitization and challenge responses) is determined by the concentration of the challenge dose, whether individuals were weakly or strongly sensitized. Similarly, the differences in the intensity of CHS response (measured as flank skin thickness) to DNCB and squaric acid dibutyl ester (SADBE) were associated with challenge doses (Scott et al., 2002). The increase in skin-fold thickness following challenge of humans or mice sensitized with increasing doses of DNCB, as well as the data that showed inverse correlation between the magnitude of sensitization dose and the dose needed to induce measurable response (Friedmann, 2007; Scott et al., 2002) demonstrated the contribution of sensitization to the intensity of CHS. Although these studies clearly demonstrated the contribution of sensitization to the intensity of CHS expression, the underlying mechanisms remain unknown.

The present study was undertaken to explore the impact of intensity of sensitization on CHS to DNCB in rats by measuring the proportion of animals that responded as well as the intensity of CHS in rats sensitized with two different DNCB concentrations (i.e. 0.4% and 4%). These doses were chosen on the basis of preliminary testing that showed that they induced the challenge responses that differ markedly both in the incidence as well as in the intensity. The impact on CHS was evaluated on the basis of the proportion of animals sensitized with these doses that responded by an increase in ear swelling and draining lymph node (cdLN) activity (cellularity, proliferation), and effectors cytokine (i.e. interferon [IFN]- γ and interleukin [IL]-17) production) to a challenge with a non-irritant dose (0.13%) of DNCB and measures of response intensities. Beside parameters of the efferent phase response, the cellularity, proliferation, and cytokine-producing activity of cells within lymph nodes that drain the skin (sdLN) sensitized with each DNCB dose was measured as well. Based on the effects of low and high doses on these parameters, assumptions about the contribution of magnitude of sensitization doses to CHS to DNCB were drawn. Experiments were performed in rats, an experimental model suitable for exploring immune mechanisms of contact allergy (Popov et al., 2012; Scheynius et al., 1988) and comparable to murine models of CHS (Dearman et al., 2002). Keeping in mind the capability of DNCB to promote predominantly a T-helper (T_H)-1 type CHS response in mice (Dearman et al., 2002), we used T_H1-prone inbred Dark Agouti (DA) rats previously shown by us to develop T_H1/Type 1 reactions to sensitization with this hapten (Popov et al., 2011, 2012). The data obtained here showed the impact of sensitization dose both on the incidence and the intensity of CHS in challenged animals and that the outcomes seemed to be related to an increase in dLN cellularity and proliferation and IL-17-producing cells particular.

Materials and methods

Chemicals

RPMI 1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 25 mM HEPES, 2 mM glutamine, 20 μ g gentamycin/ml, 50 μ M 2-mercaptoethanol (2-ME), and 5% (v/v) heat-inactivated fetal calf serum (FCS; PAA Laboratories) was used in all culture experiments. 1-Chloro-2,4-dinitrobenzene (DNCB; BDH Chemicals Ltd., Poole, UK) was dissolved in 4:1 acetone:olive oil (vehicle); 2,4-dinitrobenzenesulfonic acid (DNBS) (Aldrich Chemicals, Milwaukee, WI) was dissolved in culture medium. [³H]-Thymidine (GE Healthcare, Little Chalfont, UK) was prepared in culture medium.

Animals

Male Dark Agouti (DA) rats (8–10-weeks-old) were bred and conventionally housed at the Institute for Biological Research ‘Siniša Stanković’ (IBISS), Belgrade, Serbia. Rats were maintained in a certified facility in rooms maintained at 21–24 °C temperature control and with a 12-h light/dark cycle. All rats were provided *ad libitum* access to standard rodent chow and filtered water. Four-to-six animals were assigned for treatment group per experiment, with at least two independent experiments. Animal treatment has been carried out in compliance with the Ethical Committee of IBISS.

Experimental design

Rats were sensitized by applying 100 μ l of 0.4% or 4% (w/v) DNCB solution dissolved in acetone/olive oil (vehicle) for 2 consecutive days to the upper part ($\sim$ 16 cm²) of the dorsal skin clipped of hair. Five days later, animals were challenged by application of 50 μ l of 0.13% DNCB to the inner and outer surface of the left ear. In another set of rats, vehicle (0% DNCB) was applied to the dorsal skin and animals then challenged with 0.13% DNCB (sensitization control). For sensitization experiments, vehicle was applied to dorsal skin solely (controls). Ear swelling responses were evaluated by measuring ear thickness (in a blinded fashion) with a vernier caliper (ME-BA, Zagreb, Croatia) before and 24 h after the challenge. These values were used to calculate changes in ear thickness. Following the treatment, the rats were caged individually.

Histology

Ears skin samples were taken 24 h following the challenge and fixed in 4% buffered formalin (pH 6.9), embedded in paraffin wax for sectioning at 5 μ m, and stained with hematoxylin and eosin (H&E). All slides were then analyzed (by a certified histopathologist) using a Coolscope digital light microscope (Nikon, Tokyo, Japan).

Draining lymph node (dLN) cell preparation

Auricular lymph nodes that drain the challenged ear skin (cdLN) were harvested 24 h following elicitation. Scapular and axillary lymph nodes that drain the dorsal skin area of DNCB application for sensitization (sdLN) were harvested 5 days following the last DNCB application. Draining lymph node cells were harvested from unsensitized challenged animals (control for cdLN) or unsensitized unchallenged animals (control for sdLN) at respective timepoints. Cell suspensions were prepared by mechanical disintegration and counted by an improved Neubauer hemacytometer. The number of viable cells, determined using a trypan blue exclusion assay, always exceeded 95%.

Draining lymph node (dLN) cell culture

For proliferation measurements, freshly isolated dLN cells (0.5×10^6 /well) were cultured in 96-well microplates (Sarstedt Inc., Newton, NC) for 16 h with 0.5 μ Ci [³H]-thymidine/well added at the onset. [³H]-Thymidine incorporation was measured by liquid scintillation counting (1219 RackBeta, LKB Wallac, Turku, Finland) and proliferation expressed as counts per minute (cpm). For cytokine production, cells (5×10^5 /well) were cultured in 24-well plates (Sarstedt Inc., Newton, NC) for 72 h in the presence of medium (spontaneous production) or with 10 μ g/ml of dinitrobenzenesulfonic acid (DNBS), a water-soluble analog of DNCB (stimulated production). Cytokine production was then determined in medium conditioned by dLN cells using commercially available ELISA sets for rat IFN γ (BD Biosciences,

San Diego, CA) and mouse IL-17 (cross-reactive with rat IL-17; eBioscience, San Diego, CA). In some experiments, a commercially available ELISA set for rat IL-10 (BD Biosciences) was also used. Cytokine titers were calculated by reference to a standard curve constructed using the known amounts of respective kit-provided recombinant cytokines. The sensitivities of the IFN γ , IL-17, and IL-10 kits were 20, 16, and 16 pg/ml, respectively.

Statistics

The highest value of response obtained following the challenge of sensitization controls was used as a cut-off; all values 30% greater (at a minimum) than this value were considered positive. Results were pooled from at least two experiments and expressed as mean $\pm$ SD. All statistical analyses were performed using STATISTICA 7.0 software (StatSoft Inc., Tulsa, OK). Statistical differences between intensity of responses (e.g. ear swelling, cell number, proliferation, cytokine production) in controls vs sensitized rats were analyzed using a Mann-Whitney U-test. A difference test (difference between proportions) was used to evaluate the proportion of rats that responded to treatment (i.e. incidence of response). *p*-values <0.05 were considered significant.

Results

Choice of sensitization and challenge DNCB doses

Two DNCB doses were chosen, i.e. 0.4% and 4%, as the greatest difference in the sensitized skin draining lymph node (sdLN) cellularity and proliferation was observed with these levels in preliminary experiments. The dose of 0.13% was chosen for CHS elicitation in this study as low incidence/weak intensity of CHS response was observed in sensitized animals following challenge (see below). This dose is non-irritant, judging by the lack of difference between ear thickness in sensitization controls (unsensitized/challenged rats) and unsensitized/unchallenged (vehicle) control hosts (5.7 [$\pm$ 7.9] vs 3.0 [$\pm$ 6.2] μ m, respectively).

Effect of sensitization dose on ear swelling/challenged-skin draining lymph node activity

The challenge of animals sensitized with 0.4% DNCB resulted in ear swelling responses that were more intense than in sensitization controls (re: unsensitized/challenged animals) in only 21% of the hosts. Sensitization with 4% DNCB increased significantly the proportion of animals that responded to challenge more vigorously than sensitization controls with ear swelling and with draining lymph node cellularity, proliferation, and IL-17 production as well (Table 1). Sensitization with 0.4% DNCB resulted in numerically more intense ear swelling response in challenged animals (compared to value of response in sensitization controls),

Table 1. Proportion of DNCB-sensitized rats that responded to challenge by ear swelling and draining lymph node activity.

Parameter	Sensitization dose (%)	
	0.4	4.0
Ear-swelling response		
Ear thickness	4/19 (21%)	17/19 (89%)*
Draining lymph node cell activity		
Cell number	12/21 (57%)	19/19 (100%)*
Proliferation	13/21 (62%)	15/17 (88%)*
IFN γ production	15/21 (71%)	14/17 (82%)
IL-17 production	16/21 (76%)	17/17 (100%)*

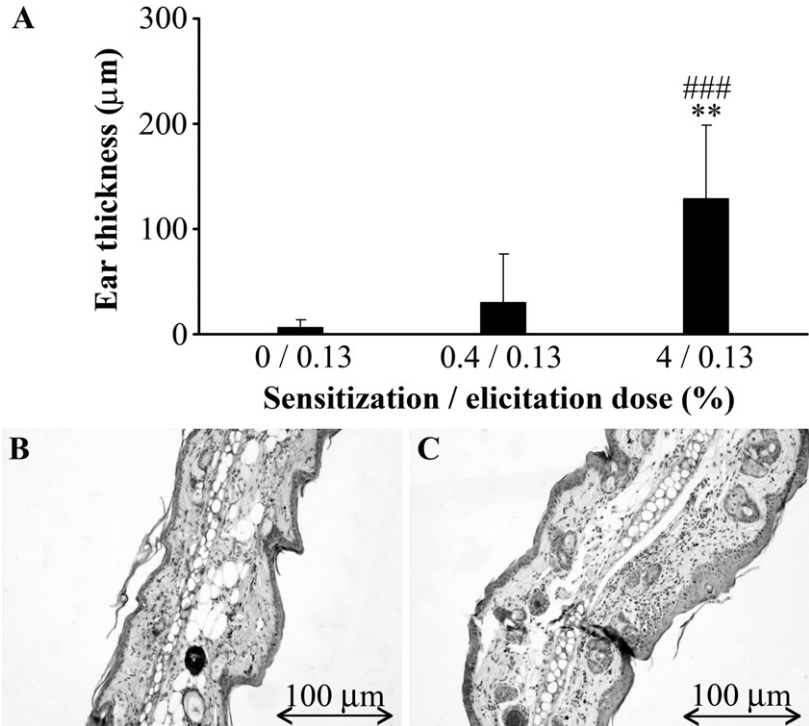
Rats were sensitized with two consecutive daily applications of 0.4 or 4% DNCB on the dorsal skin. Vehicle (acetone:olive oil) was applied to skin of sensitization controls. Rats were challenged on their ears 5 days later with 0.13% DNCB and ear-swelling responses and draining lymph node cell activity were evaluated.

Results are expressed as the proportion of sensitized rats that responded to challenge by ear swelling and by each cited draining lymph node cell activity if it was at least 30% (at minimum) above the highest value in the same response in sensitization controls.

The incidence of each of the responses in rats sensitized with 4% DNCB was compared to that in rats sensitized with 0.4% DNCB, using a difference test for proportions.

Significantly different from 0.4% sensitization regime at **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Figure 1. Ear swelling response to challenge with DNCB of sensitized rats. Rats were sensitized with two consecutive daily applications of 0.4% or 4% DNCB on dorsal skin. Vehicle (acetone:olive oil) was applied to the skin of control animals (sensitization controls). Five days later, rats were challenged with 0.13% DNCB on ears and ear swelling response was evaluated by measuring ear thickness and by histology. (a) Mean increase in ear thickness of challenged rats sensitized with 0.4% or 4% DNCB. Data are presented as mean $\pm$ SD from three-to-four independent experiments, with 4–6 rats per treatment. (b, c) Histology of ear skin of challenged rats sensitized with (b) 0.4% or (c) 4% DNCB. Value significantly different from sensitization controls (** *p* < 0.01) and challenged animals sensitized with 0.4% DNCB (### *p* < 0.001).



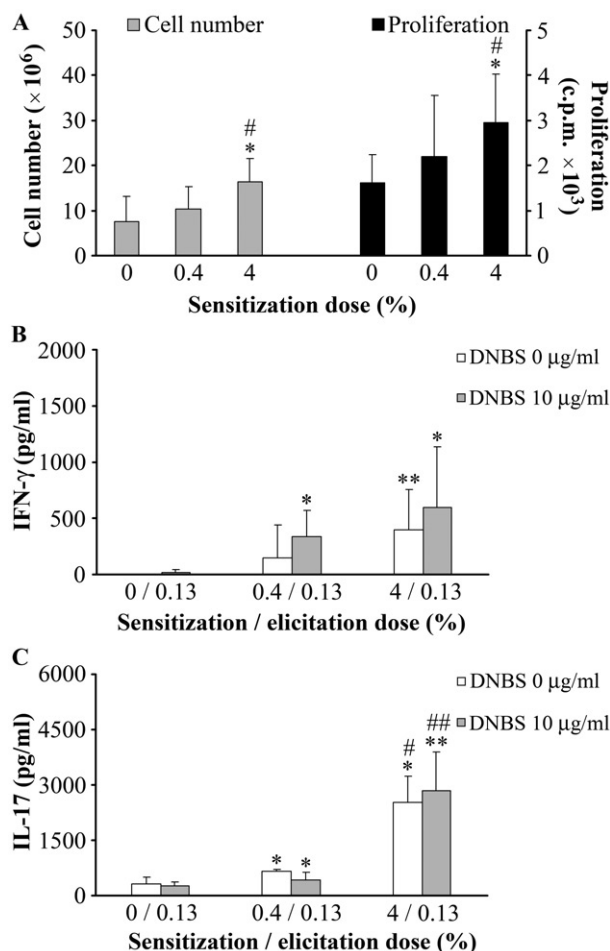


Figure 2. The activity of draining lymph nodes following challenge (cdLN). Draining auricular lymph nodes were isolated 24 h following challenge of animals sensitized 5 days earlier with 0.4% or 4% DNCB and cell numbers, proliferation, and cytokine production by harvested cells were measured. Changes in: (a) cdLN cellularity and proliferation; (b) spontaneous (0 μ g DNBS/ml) and re-stimulated (10 μ g DNBS/ml) IFN γ formation; and (c) spontaneous and re-stimulated IL-17 production, by cells from rats sensitized with 0.4% or 4% DNCB. Data are presented as mean $\pm$ SD from at least two independent experiments, with 4–6 rats per each treatment. Value significantly different from sensitization controls (* p < 0.05; ** p < 0.01) and challenged animals sensitized with 0.4% DNCB ([#] p < 0.05; ^{##} p < 0.01).

while application of 4% DNCB resulted in significantly greater responses (Figure 1a). Histological analysis revealed a more pronounced focal accumulation of mononuclear cells in challenged ears of animals sensitized with the higher DNCB dose (Figures 1b and c).

The challenge of rats sensitized with 0.4% DNCB resulted in a weak (solely numerical) increase in draining lymph node cell numbers and proliferation (compared to values seen in the sensitization controls) (Figure 2a). Significantly higher levels of these activities were noted in challenged rats sensitized with 4% DNCB. Higher (compared to sensitization controls) levels of spontaneous (significant at 4% DNCB) and DNBS-stimulated (both doses) IFN γ production by cdLN cells of sensitized rats after challenge was observed (Figure 2b); however, great variations in IFN γ production resulted in values only numerically higher in cells from rats sensitized with 4% DNCB compared to those observed in individuals sensitized with 0.4% DNCB. In contrast, a significantly greater increase in both spontaneous and re-stimulated IL-17 production by cdLN cells of rats sensitized with 4% (compared to 0.4%) DNCB was noted (Figure 2c).

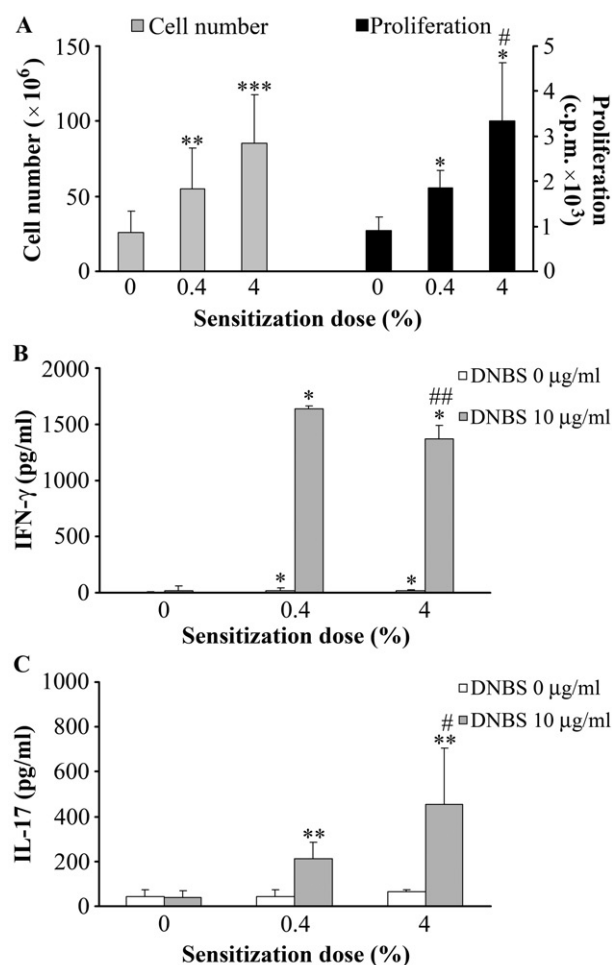


Figure 3. The activity of draining lymph nodes in rats following sensitization (sdLN). Rats were sensitized with two consecutive daily applications of 0.4% or 4% DNCB to dorsal skin. Control rats were treated with vehicle solely (controls). Five days later, draining scapular and axillary lymph nodes were harvested and total cell number, proliferation, as well as IFN γ and IL-17 production by isolated cells were measured. Changes in: (a) sdLN cellularity and proliferation; (b) spontaneous (0 μ g DNBS/ml) and re-stimulated (10 μ g DNBS/ml) IFN γ formation; and (c) spontaneous and re-stimulated IL-17 production, by cells from rats sensitized with 0.4% or 4% DNCB. Data are presented as mean $\pm$ SD from at least two independent experiments, with 4–6 rats per each of the treatments. Value significantly different from controls (* p < 0.05; ** p < 0.01, *** p < 0.001) and challenged animals sensitized with 0.4% DNCB ([#] p < 0.05; ^{##} p < 0.01).

Draining lymph node activity following sensitization with DNCB

Increases in cell numbers as well as proliferation were observed in draining lymph nodes following sensitization with 4% compared to 0.4% DNCB (Figure 3a). Low but significant increases in spontaneous IFN γ production were observed following skin sensitization with either the low or high dose of DNCB. Re-stimulation resulted in a striking increase in the amounts of cytokine produced by sdLN cells, but the level of cytokine produced was slightly, (but significantly) lower following sensitization with 4% DNCB compared to that due to 0.4% DNCB (Figure 3b). In contrast, spontaneous sdLN cell IL-17 production following sensitization with the high DNCB dose showed a tendency (p = 0.06) to be greater compared to that by cells from controls, and was significantly higher compared to levels of IL-17 produced after sensitization with the low DNCB dose (Figure 3c).

Discussion

In this study, the effect of low (0.4%) and high (4%) DNCB doses used for sensitization to the incidence as well as intensity of a CHS reaction to this hapten was examined in rats. Assumptions about the contribution of the magnitude of sensitization doses were drawn on the basis of their effect on the measured reaction parameters. The effect of each dose on the proportion of rats that responded to challenge more intensively than sensitization controls (as reflected by ear swelling and activity of lymph nodes that drain challenged as well as sensitized skin) was analyzed. The data obtained showed higher incidence and more intense efferent responses (ear swelling and dLN cellularity, proliferation and IL-17 production) in challenged animals sensitized with the high DNCB dose. Greater (compared to 0.4% DNCB) cellular proliferation and production of IL-17 by cells from lymph nodes that drain skin sensitized with 4% DNCB was observed as well.

Increases in the proportion of animals that responded to challenge more vigorously than sensitization control by ear swelling, as well as a more intense response following challenge of animals sensitized with 4% DNCB (compared to response in rats sensitized with 0.4% DNCB), suggests the dependency between rat CHS and DNCB concentration. These results are in concordance with human studies with DNCB that showed that degree of sensitization—measured as skin-fold thickness at a constant challenge dose—increased as the sensitizing dose increased (Friedmann, 2007; Schnuch et al., 2008). A low incidence of responsiveness to challenge in animals sensitized with 0.4% DNCB might have accounted for the slight (solely numerical) increase in ear swelling among the challenged animals sensitized with this dose.

Increased draining auricular lymph node cellularity and proliferation in challenged animals sensitized with 4% DNCB was in line with earlier data that demonstrated up-regulation of several T-cell activation/proliferation-related genes in the draining lymph nodes of DNFB-sensitized rats 24 h following challenge (Hartmann et al., 2006). Compared to rats that underwent the low-dose sensitization, a greater proportion of rats sensitized with 4% DNCB responded to challenge and displayed a more pronounced increase in draining auricular lymph node cellularity and proliferation; these outcomes might have contributed to the sensitization-related increase in intensity of CHS in these hosts. Compared to the rats sensitized with 0.4% DNCB, there was a lack of increase in the proportion of rats sensitized with 4% DNCB that responded to challenge by an increase in IFN γ production by dLN cells. This outcome might have been a basis for the great variations in production of this cytokine and the consequential (slightly) higher levels of IFN γ measured as compared to those attained with cells from the low-dose-sensitized rats. While the importance of this cytokine for the expression of CHS to DNFB is known in mice (He et al., 2009; Kish et al., 2009), the meaning of a lack of significant increases in IFN γ production in the challenge response to the higher DNCB dose in the rats here is unclear. Our data might, however, be in keeping with that of earlier studies that showed a self-limiting role for IFN γ in a rat CHS to DNFB (Skoglund and Scheynius, 1990; Skoglund et al., 1988).

The lack of difference between induction of IFN γ production by cdLN cells of rats sensitized with 0.4% and 4% DNCB might be a consequence of a lower cell responsivity to re-stimulation; we surmise this because similar spontaneous production of IFN γ by sdLN cells from rats sensitized with the higher DNCB dose was observed. Lower IFN γ production rates after sensitization with 4% (compared to 0.4%) DNCB seemingly did not result from differential production kinetics as (similar to what was seen in the

Day 5 data) lower levels of IFN γ were found in cultures of dLN cells 3 days post-sensitization (i.e. 3583.4 [$\pm$ 431.6] and 2736.5 [$\pm$ 360.6] pg/ml at 0.4% and 4% DNCB, respectively, compared to 112.0 [$\pm$ 194.0] by control rat cells). These particular outcomes could possibly be ascribed to the increase in production of IL-10, an anti-inflammatory cytokine that is known to inhibit IFN γ -based (i.e., T_H1) responses (Trinchieri, 2007). Indeed, significantly higher production of this cytokine by draining lymph node cells was observed 5 days following skin sensitization with 4% DNCB (142.5 [$\pm$ 20.6] pg/ml) compared to 12.5 [$\pm$ 3.5] pg/ml with 0.4% DNCB. Although production of IFN γ on a per-cell basis was lower following re-stimulation, overall, high levels of this cytokine could be achieved in the lymph node microenvironment where it is needed for development of effector (i.e. IFN γ -producing) cells.

A greater proportion of rats (compared to those that received the low-dose sensitization) sensitized with 4% DNCB responded to challenge with an increase in draining auricular lymph node cell IL-17 production, as well as higher levels of production on a per-cell basis. This type of outcome indicated that the expression of contact hypersensitivity was closely related to the sensitization dose. The greater capacity to produce IL-17 by dLN cells from rats sensitized with 4% DNCB (compared to that by cells from rats sensitized with 0.4% DNCB) supports our conclusion concerning the impact of sensitization on this cytokine during the CHS response to DNCB. Increases in IL-17 production during sensitization, as well as in the challenge response, might have a strong impact on the severity of CHS. The importance of this cytokine for murine CHS response to dinitrohalobenzenes is known (see He et al., 2006, 2009; Nakae et al., 2002) and it is considered a central mediator of skin inflammation in cutaneous allergic reactions (Pennino et al., 2010).

In conclusion, the increase in the proportion of animals sensitized with the higher DNCB dose that responded to challenge with an increase of dLN activity (cellularity, proliferation, and IL-17-producing cells) suggests that the impact of the sensitization dose might rely on these variables. Although assumptions about the contribution of magnitude of sensitization dose to CHS were made on the basis of the effect of only two doses (i.e. low and high DNCB), coincidental changes in these dLN activities in challenged and sensitized animals support such considerations.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the contents and writing of the paper.

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