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Strain differences in contact hypersensitivity reaction to dinitrochlorobenzene (DNCB) in rats

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ABSTRACT

Genetic factors are among the most important determinants of susceptibility to induction of allergic contact dermatitis. A limited number of studies of experimental contact hypersensitivity (CHS) in animals has shown differences in the severity of CHS; however, the underlying mechanisms are unknown. In this study comparative analysis of CHS to low and high dinitrochlorobenzene (DNCB) doses regimen of sensitization/challenge in inbred Dark Agouti (DA) and Albino Oxford (AO) rats was examined. Basic aspects of draining lymph node (dLN) activity (cellularity, proliferation), proinflammatory (IFN-γ, IL-17) and anti-inflammatory (IL-10) cytokine gene expression and production, as well as IL-12 and IL-23 subunits mRNA expression, were examined in challenge and sensitization phase of CHS reaction. Lower (compared to DA) intensity of CHS in AO rats was associated with lack of (or negligible) dLN responses in challenge phase (*ex vivo*, hapten- or IL-2-stimulated cell proliferation and proinflammatory cytokine mRNA and production levels) but with lack of changes in IL-10 response. Less pronounced dLN activity of sensitized animals of this strain was observed as well. Higher proliferative activity and more pronounced proinflammatory cytokine response during challenge and sensitization phase suggest these activities as underlying mechanisms of higher susceptibility of DA rats to CHS response to DNCB.

1. Introduction

Allergic contact dermatitis (ACD) is a common T-cell mediated skin inflammatory disease in humans, that develops at the site of skin re-exposure to contact allergens in individuals previously sensitized with the same agent (Saint-Mezard et al., 2004). Contact hypersensitivity (CHS) to haptens, animal model of ACD, has provided a great contribution to understanding pathophysiology of this disease. Two distinct phases are necessary to achieve optimal CHS reaction: the sensitization phase (known as afferent or induction phase) starting with the first contact of skin with hapten and challenge phase (known as efferent or elicitation phase) which occurs following second hapten encounter (Grabbe and Schwarz, 1998; Kish et al., 2009). While sensitization phase has no clinical manifestations in the majority of cases, challenge of skin of sensitized individuals leads to local inflammatory reaction. CHS is considered usually as Th1/type1 immune reaction, with IFN-γ as main effector cytokine (Gocinski and Tigelaar, 1990; Xu et al., 1996). Recent studies of ACD in humans and CHS in mice have stressed importance of both Th1/type1 and Th17/type17 cells for full development of reaction (He et al., 2009; Pennino et al., 2010).

Despite the fact that limited studies exist in humans, genetic factors (along with dermal integrity), beside environmental factors (Schnuch and Carlsen, 2011), are considered as most important determinants of susceptibility to induction of ACD (Felter et al., 2002). Concerning CHS, there are only few studies that have examined genetically determined differences of susceptibility to haptens in inbred strains of experimental animals. It was shown that more severe and mild intensity of CHS reaction to dinitrofluorobenzene (DNFB) in C57BL/J and BALB/C mice, respectively, correlated with proinflammatory effects of the hapten in challenged skin of these strains (Bonneville et al., 2007). Studies in rats with different propensity of development of Th1/Th2 cytokine responses showed that Th1-prone Lewis (Lew) (RT-1^l MHC haplotype) rats were more sensitive to dinitrohalobenzenes, compared to Th-2 prone Brown Norway (BN) rats (RT-1^n MHC haplotype), based on microscopic and macroscopic evaluation of challenged skin (Peszkowski et al., 1994). Cellular and molecular mechanisms underlying such differences in CHS expression, however, remained largely unknown.

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Studies of genetic factors that are involved in differences related to development of inflammatory/immune mediated diseases in these rats showed both MHC-dependent (due to a deficit in $CD8^+$ cells in BN rats) as well as MHC-non-dependent differences (predominance of type 2 and regulatory cytokines, higher representation of $CD45RC^{low}$ cells) (Fournié et al., 2001).

Using Dark Agouti (DA, $Rt1^{av1}$) rats the proinflammatory character (IFN-γ and IL-17 response by draining lymph node cells) of sensitization phase of CHS to low DNCB dose (0.4% w/v) was previously shown (Popov et al., 2011). Lower intensity of proinflammatory cytokine production resulted from preliminary experiments in that study in Albino Oxford (AO RT^u) rats indicated differences in CHS between these two rat strains. Studies of immune-inflammatory disease such as experimental autoimmune encephalomyelitis (EAE) in DA and AO rats showed that major differences which caused susceptibility to disease were mainly related to type 1 (Lukic et al., 2001; Mostarica-Stojković et al., 1982) and Th17 cytokines (Markovic et al., 2009). In the light of these reports, the aim of this study was comparative analysis of immune/inflammatory responses in CHS to DNCB in these two strains. Low and high DNCB doses were used and cardinal aspects of activity of lymph nodes that drain challenged as well as sensitized skin were analyzed. It was shown that lower intensity of CHS in AO rats was associated with lack (or low levels) of reactivity of draining lymph nodes to challenge (proliferative activity and inflammatory cytokine gene expression and production). Less pronounced draining lymph node response to sensitization was observed in this rat strain.

2. Materials and methods

2.1. Chemicals and reagents

One-chloro-2,4-dinitrobenzene (DNCB) was obtained from BDH Chemicals Ltd., Hull, UK and dissolved in 4:1 acetone:olive oil (vehicle). RPMI-1640 culture medium (PAA Laboratories, Pasching, Austria) supplemented with 25 mM HEPES, 2 mM glutamine, 20 mg/ml gentamycine, 5 μg/ml antifungal agent voriconazole and 5% (v/v) heat-inactivated fetal calf serum (FCS) (PAA Laboratories) was used in cell culture experiments. Two, four-Dinitrobenzenesulfonic acid (DNBS) (Aldrich Chemical Company Inc., Milwaukee, USA), radiolabeled [^{3}H]-thymidine (GE Healthcare, UK), recombinant human IL-2 (rIL-2, R&D Systems, Minneapolis, USA) and Concanavalin A (ConA) (Pharmacia, Uppsala, Sweden) were dissolved in culture medium, and added in final concentration 10 μg/ml, 50 μCi/ml, 50 i.j./ml and 1 μg/ml, respectively. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (ICN Biomedicals Inc, Aurora, USA) was dissolved in phosphate-buffered saline (PBS, pH 7.2). FITC-labeled mouse antibodies to rat CD4, PE-labeled mouse antibodies to rat CD8 and PE-labeled mouse antibodies to rat CD25 (IgG1) were purchased from eBioscience Inc. (San Diego, CA, USA). Propidium iodide and RNase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Ten to twelve week old male Dark Agouti (DA) and Albino Oxford (AO) rats, bred and conventionally housed at the Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia, were used. They were maintained at 12 h photoperiod, 21/24 °C temperature control and 60% relative humidity. All rats had *ad libitum* access to standard rodent chow and water throughout the study. Four to six animals were assigned to each treatment group per experiment, in at least two independent experiments. After treatment, animals were caged individually. All animal procedures were complied with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee of the Institute for Biological Research "Sinisa Stankovic".

2.3. Contact hypersensitivity

Animals were sensitized by applying 100 μl of solution of 0.4% (w/v) or 4% (w/v) DNCB dissolved in acetone/olive oil (vehicle) for two consecutive days to the upper part (~16 cm^2) of the dorsal skin clipped of hair. Five days following the last application of DNCB, rats were challenged to the ventral and dorsal surfaces of the right ear by an application of 50 μl of three times lesser dose of DNCB. Rats sensitized with 0.4% DNCB were challenged with 0.13% DNCB (low dose sensitization/challenge regimen), while rats that were sensitized with 4% DNCB were challenged with 1.3% DNCB (high dose sensitization/challenge regimen). Control rats received vehicle (acetone/olive oil) both to the dorsal skin area and to the ears. One group of non-sensitized animals were challenged with 0.13% DNCB (0%/0.13%) or 1.3% DNCB (0%/1.3%), as additional controls. The ear swelling, an *in vivo* measure of contact hypersensitivity (CHS) response, was assessed in a blinded fashion by measuring pinnal thickness 24 hours following ear challenge, by a hand-held engineer's micrometer (six measurements per ear). In separate experiment, ear thickness was measured 48 hours following ear challenge. An increase in ear swelling was defined as a difference between ear thickness 24 hours or 48 hours after challenge and ear thickness before challenge.

2.4. Histology

Ears skin samples were taken 24 hours or 48 hours following challenge. Dorsal skin was taken three and five days following sensitization. Tissue samples were fixed in 4% buffered formalin (pH 6.9), embedded in paraffin wax for sectioning at 5 μm, and stained with hematoxylin and eosin (H&E). Pathohistological analyses were made (by a certified specialist) using a Coolscope digital light microscope (Nikon, Tokyo, Japan). Epidermal and dermal infiltrate of mononuclear cells (MNC) and polymorphonuclear cells (PMNC) was counted in at least 30 non-overlapping microscopic fields under immersion, covering the entire area of the section. Area of one field of view corresponded to 0.196 mm^2, at 1000× magnification (10× ocular/100× objective). The average cell number per field, determined for every animal, was used for statistical analysis, and the results were presented as median and range.

2.5. Draining lymph node (dLN) cell preparation

Auricular lymph nodes (that drain the challenged ear skin) were harvested 24 hours or 48 hours following challenge. Suprascapular and axillary lymph nodes (that drain sensitized skin) were isolated five days following sensitization. Lymph nodes were also harvested from control animals. Cell suspensions were prepared by mechanical teasing of dLN over nylon mesh (70 μm nylon, BD Bioscience, Bedford, USA) and cells were counted by the improved Neubauer hemacytometer. The number of viable cells, determined by using a trypan blue exclusion assay, always exceeded 95%.

2.6. Draining lymph node cell proliferative activity

For proliferation measurements, freshly isolated dLN cells (0.6×10^6/well) were cultured in 96-well microplates (Sarstedt Inc., Newton, NC, USA) for 16 hours with 0.5 μCi [^{3}H]-thymidine/well added at the onset of culture (*ex vivo* proliferation). For IL-2-stimulated proliferative activity dLN cells (0.6×10^6 cells/well) were cultured in the culture medium solely or in the presence of human rIL-2 (50 i.u./ml), with 0.5 μCi [^{3}H]-thymidine/well added following 24 hours of culture and incubated for the next 16 hours. For hapten-stimulated proliferative activity dLN cells (0.6×10^6 cells/well) were cultured in the culture medium solely or in the presence of DNBS (10 μg/ml) for 96 hours, with 0.5 μCi [^{3}H]-thymidine/well added during the last 16 hours of culture. Incorporation of [^{3}H]-thymidine was measured by liquid scintillation counting (1219 RackBeta, LKB Wallac, Turku, Finland) and proliferation expressed as counts per minute (c.p.m.).

2.7. Analysis by flow cytometry

Draining lymph node cells (1×10^6) were incubated on ice with FITC- and PE-conjugated antibodies to rat CD4 and CD8, respectively, or PE-conjugated antibodies to rat CD25 for 30 minutes. After washing, the cells were fixed with 1% paraformaldehyde and assayed for fluorescence intensity on a FACSCalibur cytometer (BD Biosciences, Heidelberg, Germany).

2.8. MTT assay for draining lymph node cell metabolic viability

For assessing the dLN cell viability, MTT reduction assay described for lymphocyte cell lines (Denizot and Lang, 1986) was employed. Cells (1×10^5 cells/well of 96-well plate) were placed in wells with culture medium. MTT (final concentration 0.5 mg/ml) was added immediately and incubated with cells for 3 hours at 37 °C in a humidified atmosphere with 5% CO_2. Produced formazan was dissolved by overnight incubation in acidified SDS (10% SDS–0.01 N HCl). Extracted chromogen was quantitated spectrophotometrically at 540 nm using ELISA 96-well plate reader (Shimadzu Corporation, Lakewood, USA).

2.9. Analysis of hypodiploid cells in draining lymph node cell suspensions by propidium iodide and flow cytometry

For measuring dLN cell apoptosis, the presence of hypodiploid cells was analyzed by staining with propidium iodide and flow cytometry analysis as described (Riccardi and Nicoletti, 2006). Cells (2×10^6) were washed in PBS, and then fixed by adding 70% (v/v) cold ethanol (in PBS), for at least 30 minutes on ice. Cells were then washed twice in PBS, at 900× *g*, re-suspended in 1 ml of staining solution (10 μg/ml of propidium iodide and 51 μg/ml RNase, all in PBS) and incubated for 45 minutes at room temperature in the dark. Cells were analyzed on FACSCalibur cytometer (BD Biosciences), collecting 20,000 events. Apoptotic nuclei appeared as a broad