



Research paper

Complementary methods for contact hypersensitivity (CHS) evaluation in mice

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ABSTRACT

Contact hypersensitivity (CHS) is an experimental model of allergic contact dermatitis (ACD) that can be studied in mice. For CHS responses, mice are immunized by painting with a reactive hapten, such as 1-fluoro-4,6-dinitrobenzene (DNFB), on the shaved abdominal and chest skin. Subsequently, the ears are challenged with diluted hapten, eliciting 'hypersensitive' ear-swelling reactions, which can be measured with a micrometer. In this manuscript we present complementary methods that can be used to evaluate CHS in mice that include: ear weight, vascular permeability, myeloperoxidase (MPO) activity, IFN- γ concentration in ear extracts and also IFN- γ production by auricular lymph node cells (ELNC). The biochemical evaluation of CHS can be also supported by proliferation assay, measurement of IFN- γ production by skin-draining lymph node cells employing ELISA test and by evaluation of IFN- γ ⁺ TCR $\alpha\beta$ CD8 cells with the use of flow cytometry.

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1. Introduction

Allergic contact dermatitis (ACD) is a common skin disease that is caused by type IV delayed-type hypersensitivity responses to antigens that come into contact with the skin (Nosbaum et al., 2009). Contact hypersensitivity (CHS) is an experimental model of ACD that can be studied in mice (Christensen and Haase, 2012). CHS responses can be mediated by either CD4⁺ Th1, MHC class II-restricted cells locally

releasing IFN- γ to recruit a characteristic inflammatory infiltrate (Askenase et al., 2004), or by CD8⁺ MHC class I-restricted Tc1 cells that can similarly release IFN- γ , but predominantly mediate cytotoxic damage to local skin cells such as keratinocytes (Kalish and Askenase, 1999; Gocinski and Tigelaar, 1990) and now also IL-17 producing Th17 cells (Zhao et al., 2009; He et al., 2009). More recently it was shown that liver NK cells can play the role of CHS effector cells (O'Leary et al., 2006; Paust et al., 2010).

CHS can be induced by topical skin immunization with reactive chemical haptens dissolved in organic solution. Tc1-mediated CHS induced via skin immunization with DNFB belongs to most frequently studied animal models of skin hypersensitivity (Askenase et al., 2011). Contact hypersensitivity is divided into inductive and effector limbs. The inductive limb results from hapten immunization and takes about 4–5 days. After painting of the chest and the abdominal skin with the hapten, it penetrates through the stratum corneum barrier of the skin. During this step the sensitizer binds to the self proteins in the skin (Kaplan et al., 2012). Then, hapten-conjugated self proteins are recognized

Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; DDC, dermal dendritic cells; DNFB, 1-fluoro-4,6-dinitrobenzene; DNP, 2,4-dinitrophenol; DNP-BSA, 2,4-dinitrophenylated bovine serum albumin; ELNC, auricular lymph node cells; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IFN- γ , interferon-gamma; IL-1 β , interleukin 1-beta; IL-17, interleukin 17; LNC, lymph node cells; MHC, major histocompatibility complex; MPO, myeloperoxidase; NK, natural killer cells; NKT, natural killer T lymphocytes; OD, optical density; SPF, specific pathogen free; Tc1, type 1 cytotoxic lymphocytes; Th1, type 1 T helper lymphocyte

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by innate immune mechanisms in the skin that results in the induction of pro-inflammatory cytokines such as IL-1 β (Sutternvala et al., 2006; Martin et al., 2011). This leads to the activation of dermal dendritic cells (DDC). The activated DDC that are directly haptenated or picked up hapten-conjugated self proteins migrate to the draining lymph nodes, where they induce CHS-effector T cells which are ready to play their effector function within 4–5 days post sensitization (Kaplan et al., 2012). Additionally, within 1 day post hapten immunization liver NKT and peritoneal B-1 B cells are activated and then participate in the recruitment of CHS-effector T cells (Askenase et al., 2011; Kerfoot et al., 2008). The role of NKT and IL-4 and B cells in CHS initiation was confirmed by experiments employing CD1d $^{-/-}$, J α 18 $^{-/-}$, IL-4R $\alpha^{-/-}$, STAT6 $^{-/-}$ and pan-B cell deficient JH $^{-/-}$ mice respectively (Askenase et al., 2011).

The effector limb is elicited, following secondary challenge with the same hapten. It was previously shown that effector limb of CHS has biphasic nature and is divided into two phases (Askenase et al., 2004). First, an elicited initiating phase very early after hapten challenge, and, second, a classical late inflammatory phase mediated by the recruited CHS-effector T cells. The elicited early component peaks at 2 h following the hapten challenge and is due to an “initiation process” that results in local recruitment of antigen-specific effector T cells to the site of elicitation e.g. the ears to begin the late phase (Askenase et al., 2004). The CHS-effector T cells release pro-inflammatory cytokines such as IFN- γ and start killing the hapten-labeled cells e.g. keratinocytes which results in edema – ear swelling that can be measured with a micrometer 24 h after the challenge (Askenase et al., 2004).

The aim of this paper is to present objective laboratory methods that may help to confirm ear swelling measurements with a micrometer.

2. Materials and methods

2.1. Mice

Specific pathogen free (SPF) male BALB/c (H-2^d) mice were from the breeding unit of the Department of Medical Biology, Jagiellonian University College of Medicine. Mice were rested for at least 1 week before use, maintained under specific SPF and used at 6–10 weeks of age in groups of 5–6. All experiments were conducted according to guidelines of Jagiellonian University College of Medicine. All experiments were repeated at least three times, and a representative experiment is shown in the figures.

2.2. Reagents

1-fluoro-4,6-dinitrobenzene (DNFB), Evans blue, formamide, hexadecyltrimethyl-ammonium bromide and o-dianisidine dihydrochloride were obtained from Sigma, St. Louis, MO. IFN- γ BD OptEIA Set was from BD Biosciences, San Diego, CA. 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) was from Biosearch Technologies, Inc., Novato, CA.

2.3. Immunization and elicitation of CHS

Unanesthetized mice were contact sensitized with 25 μ l of 0.5% DNFB in acetone: olive oil (4:1) applied by painting on the

shaved abdominal and chest skin (positive control) or sham immunized by painting with 25 μ l of vehicle alone (negative control). Five days later, CHS responses were elicited by painting both ears with 5 μ l of 0.1% DNFB in acetone and olive oil (1:1) (Askenase et al., 2011; Kerfoot et al., 2008). Resulting ear thickness was measured prior to testing with a micrometer (Mitutoyo, Tokyo, Japan) by an observer unaware of the experimental groups and then again at 24 h after challenge. Increased ear thickness was expressed as the mean $\pm$ SE.

2.4. Measurement of ear weight

To determine ear weight animals were sacrificed 24 h after challenge. The ears were removed and 6 mm diameter punch in central part of each ear was made with biopsy punch (Frey Products Corp., cat# BP60). The weight of the punches was measured with the use of analytical laboratory scale (Sartorius).

2.5. Vascular permeability test

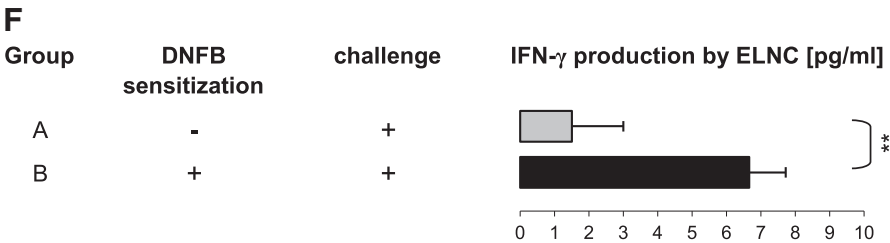
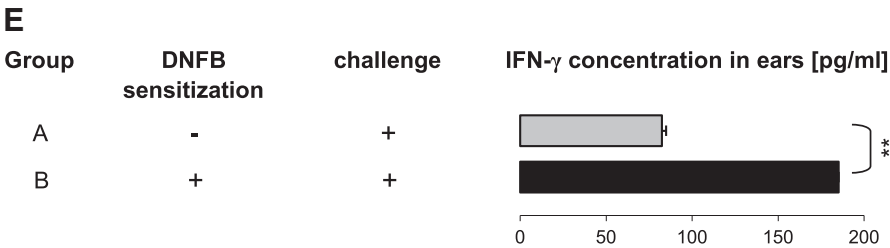
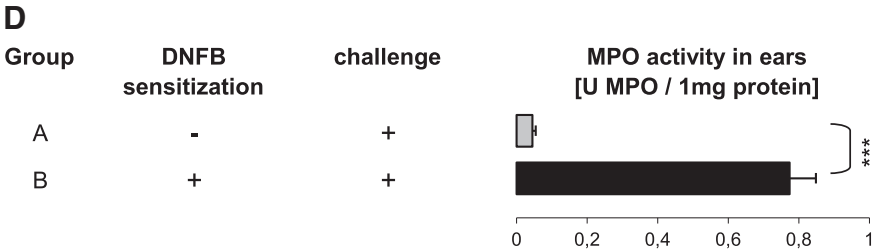
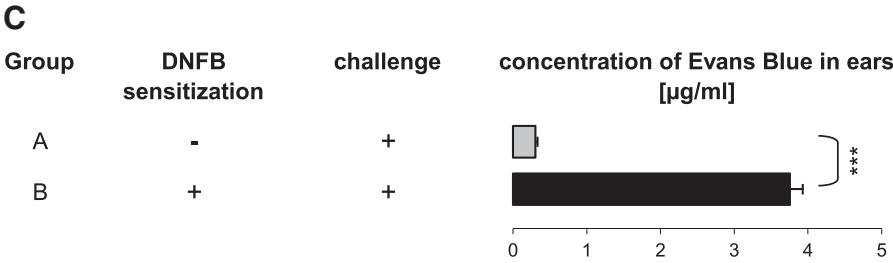
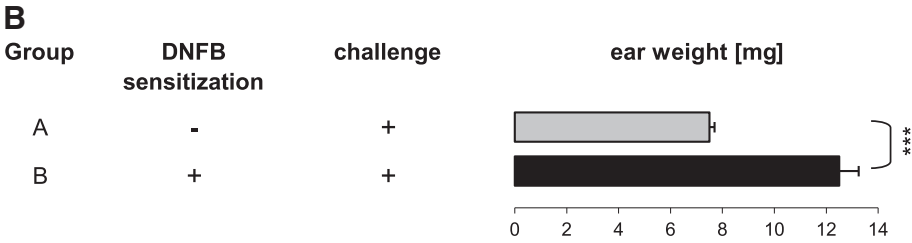
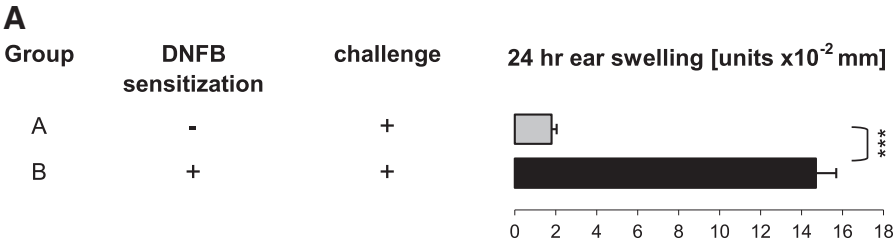
To assess very early changes in vascular permeability, DNFB immune or naïve mice were challenged with 5 μ l of 0.1% DNFB and injected with 1% Evans blue dye (83 μ g/g body weight) 23 h later. 1 h after Evans blue injection mice were anesthetized and sacrificed. Ears were removed and 6 mm diameter punch in central part of the ear was made with biopsy punch (Frey Products Corp., cat# BP60). Ear punches were transferred to tubes containing 1 ml of formamide. After 18 h incubation at 37 °C the samples were centrifuged and the optical density (OD) of the supernatant fluids was read at 565 nm against blank containing formamide alone (Majewska-Szczepanik et al., 2012).

2.6. In vitro measurement of IFN- γ in CHS ear extracts

To determine local production of IFN- γ in elicited DNFB CHS, mice were immunized with 0.5% DNFB or sham sensitized and 5 days later challenged with 5 μ l of 0.1% DNFB. Ears were removed 24 h post challenge and 6 mm diameter punch in central part of the ear was made. The biopsies were collected from the distal site of CHS ear responses and were frozen rapidly in liquid N₂ and then were subsequently thawed and extracted in 300 μ l cold PBS on ice with a tissue micro-homogenizer. Concentration of IFN- γ was measured by ELISA with the use of BD OptEIA Set (BD Biosciences, San Diego, CA) (Majewska-Szczepanik et al., 2012).

2.7. Myeloperoxidase (MPO) assay

Neutrophil infiltration to the inflamed ears was indirectly quantitated using an MPO assay, as described previously (Szczepanik et al., 2000). Ears were removed 24 h post challenge and 6 mm diameter punch in central part of the ear was made. The biopsies were collected from the distal site of CHS ear responses and were homogenized in 0.5% hexadecyltrimethylammonium bromide pH = 6.0 (50 mg of tissue/ml). The homogenates were freeze-thawed 3 times, centrifuged at 40,000 g and then 20 μ l aliquots were mixed with 200 μ l phosphate buffer (pH = 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 5 $\times$ 10^{−4} % H₂O₂ and



incubated at 25 °C for 20 min. The absorbance was measured at 460 nm in 96-well flat bottom plates. MPO activity was expressed in units per protein concentration (U/g of protein).

2.8. T cell proliferation assays

BALB/c mice were painted with either 0.5% DNFB or vehicle alone. Five days later inguinal and axillary lymph nodes were isolated and 3×10^5 LNC were cultured in 96-U bottom microtiter plates in triplicate in the presence of three fold dilutions of DNP-BSA in 200 μ l of RPMI 1640 containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 25 mM Hepes, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum for 48 h prior to the addition of [^{3} H]-thymidine for an additional 18 h. [^{3} H]-thymidine incorporation was determined by β liquid scintillation counting (Ptak et al., 2009). Results are presented as mean $\pm$ SE.

2.9. Measurement of IFN- γ in culture supernatants

To determine IFN- γ production, BALB/c mice were sensitized with 0.5% DNFB or sham immunized as described in [Materials and methods](#). Five days post immunization axillary and inguinal lymph node cells were collected and processed under aseptic conditions.

Lymph node cells (3×10^6) were cultured in 1 ml RPMI 1640 medium supplemented with 10% FCS in the presence of 100 μ g/ml DNP-BSA. Cells were distributed in triplicate wells in flat 24 well Falcon plates. After 48 h culture supernatants were collected and then tested for IFN- γ concentration with the use of BD OptEIA Set (BD Biosciences, San Diego, CA) (Majewska-Szczepanik et al., 2012). In some experiments 24 h post ear challenge with 0.1% DNFB, auricular lymph nodes (ELN) were isolated from sham or DNFB immunized mice. Then auricular lymph node cells (ELNC) were cultured in the presence of DNP-BSA as described above and culture supernatants were tested for IFN- γ concentration.

2.10. Staining for intracellular IFN- γ

For intracellular cytokine staining, lymph node cells (5×10^6) were cultured for 5 h with monensin solution (BD Biosciences, Franklin Lakes, NJ), PMA 50 ng/ml and ionomycin 500 ng/ml both from Sigma Chemical Co., St. Louis MO. Then, cells were stained for surface markers with anti-TCR β -FITC and anti-CD8a-PerCP-Cy5.5 mAbs (BD Biosciences, Franklin Lakes, NJ), and further processed using a fixation buffer and permeabilization buffer both from eBioscience (eBioscience, San Diego, CA). Cells were then stained with anti-IFN- γ -APC (BD Biosciences, Franklin Lakes, NJ). Analysis was performed on a FACS Calibur flow cytometer using CellQuest software.

2.11. Statistics

Data in graphs are shown as mean $\pm$ SE. ANOVA followed by Student's *t* test with Bonferroni correction was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Ear swelling correlates with increased ear weight, vascular permeability, MPO activity and IFN- γ concentration in ear extracts

BALB/c mice were contact sensitized with 0.5% DNFB on the shaved abdominal and chest skin (positive control) or sham immunized by painting with vehicle alone (negative control). Five days later, CHS responses were elicited by painting both ears with 0.1% DNFB. Animals were tested for CHS with a micrometer 24 h after ear challenge.

Data presented in [Fig. 1A](#) show that mice skin sensitized with DNFB and challenged 5 days later on the ears develop significant ear swelling when compared with negative control mice that were sham immunized and then similarly challenged (Group B vs A).

The ear swelling data were fully confirmed in further experiments showing that increase of ear edema measured with the micrometer correlates with augmented ear weight

Fig 1. Complementary methods that confirm ear swelling. (A) Evaluation of contact hypersensitivity (CHS) to DNFB with a micrometer. BALB/c mice were contact sensitized by abdominal skin painting with 0.5% DNFB or sham sensitized with vehicle alone. Then on day 5, ear swelling responses were elicited by contact painting of both ears with 5 μ l of 0.1% DNFB in vehicle in mice contact sensitized with DNFB (Group B) vs sham sensitized mice (Group A), and measuring ear thickness with a Mitutoyo micrometer 24 h after challenge. Ear swelling was expressed as mean $\pm$ SE. $n = 12$. *** $p \leq 0.001$. (B) Ear swelling correlates with ear weight. To determine ear weight the ears were removed 24 h after challenge and 6 mm diameter punch of each ear was made with biopsy punch (Frey Products Corp., cat# BP60). The weight of the punches was measured with the use of analytical laboratory scale. Ear weight was expressed as mean $\pm$ SE. $n = 12$. *** $p \leq 0.001$. (C) Observed tissue edema is a consequence of increased vascular permeability. To assess changes in vascular permeability, DNFB (Group B) vs sham (Group A) sensitized BALB/c mice were ear challenged with 5 μ l of 0.1% DNFB and injected with 1% Evans blue dye. One hour after injection of Evans blue mice were killed and 6 mm diameter punch of the ear was made. Evans blue dye was extracted into formamide and the optical density (OD) was read at 565 nm against blank containing formamide. Results shown as mean $\pm$ SE. $n = 10$. *** $p \leq 0.001$. (D) Increased activity of MPO in tissue extracts corresponds to inflammation in the ears. BALB/c mice were sensitized with DNFB (Group B) or sham immunized (Group A) and challenged 5 days later. Ears were removed 24 h post challenge and 6 mm diameter punch of the ear was made. Biopsies were homogenized in 0.5% hexadecyltrimethyl ammonium bromide. Then homogenate aliquots were mixed with phosphate buffer containing o-dianisidine dihydrochloride and H_2O_2 and incubated at 25 °C for 20 min. The absorbance was measured at 460 nm. MPO activity was expressed in units per protein concentration (U/g of protein). Results shown as mean $\pm$ SE. $n = 10$. *** $p \leq 0.001$. (E) Increased concentration of IFN- γ in ear extracts of DNFB sensitized mice. BALB/c mice were sensitized with DNFB (Group B) or sham immunized (Group A) and underwent subsequent challenge 5 days post immunization. 24 h after challenge ears were removed and 6 mm diameter punch of the ear was made. The biopsies were frozen rapidly in liquid N_2 and were subsequently thawed and extracted in 300 μ l cold PBS on ice with a tissue microhomogenizer. Concentration of IFN- γ was measured by ELISA with the use of BD OptEIA Set (BD Biosciences, San Diego, CA). Results shown as mean $\pm$ SE. $n = 10$. ** $p \leq 0.01$. (F) IFN- γ production by auricular lymph node cells (ELNC). BALB/c mice were skin sensitized with 0.5% DNFB (Group B) or sham immunized (Group A) as described in [Material and Methods](#). Five days later both groups of mice were ear challenged with 0.1% DNFB. 24 h post challenge ELN were collected and processed under aseptic conditions and then 3×10^6 ELNC were cultured in the presence of 100 μ g/ml DNP-BSA. Cells were distributed in triplicate wells in flat 24 well Falcon plates. After 48 h culture supernatants were collected and then tested for IFN- γ concentration. Results are presented as mean $\pm$ SE. $n = 4$. ** $p \leq 0.01$.

(Fig. 1B) and vascular permeability (Fig. 1C). Additionally, increased ear swelling strongly correlated with myeloperoxidase (MPO) activity (Fig. 1D) and IFN- γ concentration (Fig. 1E) in ear extracts.

Additionally, IFN- γ production by auricular lymph node cells (ELNC) correlates with both ear swelling and IFN- γ concentration in ear extracts (Fig. 1F; Group B vs A).

3.2. Contact hypersensitivity in vivo correlates with in vitro lymph node cell proliferation and IFN- γ production

Contact hypersensitivity reaction as an example of T cell mediated immune response can be also tested in vitro by evaluation of lymph node cell proliferation and cytokine production. To test CHS in vitro BALB/c mice were DNFB or sham sensitized. Five days later LNC were cultured in the presence of DNP-BSA antigen as described in [Materials and methods](#) and then culture supernatants were tested for IFN- γ concentration whereas, LNC pulsed with [^{3} H]-thymidine were evaluated for cell proliferation.

Data presented in Fig. 2A show increased [^{3} H]-thymidine incorporation by DNFB immune LNC when compared with LNC from sham sensitized mice (Group B vs A). Similarly, LNC from DNFB sensitized mice produced significantly higher level of IFN- γ when compared with LNC isolated from sham immunized mice (Fig. 2B; Group B vs A).

Additionally, flow cytometry experiments showed increased percentage of IFN- γ^+ TCR $\alpha\beta$ CD8 lymphocytes in axillary and inguinal lymph node cells isolated from DNFB sensitized mice when compared with sham immunized animals (Fig. 2C; Group B vs A).

4. Discussion

Contact hypersensitivity (CHS) is induced by haptens that conjugate to self-protein antigens in the skin. CHS is mediated by local extravascular recruitment of antigen-specific circulating CHS-effector T cells that cause inflammatory tissue swelling, peaking 24 h after secondary skin challenge with the same hapten ([Asherson and Ptak, 1968](#)). As was shown by van Loveren et al. the effector phase of CHS can be detected with the aid of various micrometers by the measurement of the ear swelling ([Van Loveren et al., 1984](#)). The authors found that various micrometers measure ear swelling with varying accuracy. The slide caliper applies a lot of pressure and therefore measures a small late component in comparison with other instruments. The spring caliper that applies the least pressure to the ears seems to give the most accurate measurements of the actual thickness of the ears ([Van Loveren et al., 1984](#)). However, some of the micrometers that apply more pressure e.g. Mitutoyo micrometer more likely compress the fluid that accumulates in the early phase of edema formation. Thus, these micrometers tend to reveal more clearly, as the result of the pressure they apply, the biphasic nature of CHS that cannot be appreciated as readily with the light-pressure spring caliper. As the choice of the proper micrometer is half of success, experience ensures accurate measurements ([Van Loveren et al., 1984](#)). The ear swelling is measured by an observer unaware of the experimental groups but still the readings can be influenced by subjective feelings especially when tests are performed by an inexperienced experimenter. In order to confirm ear swelling readings other

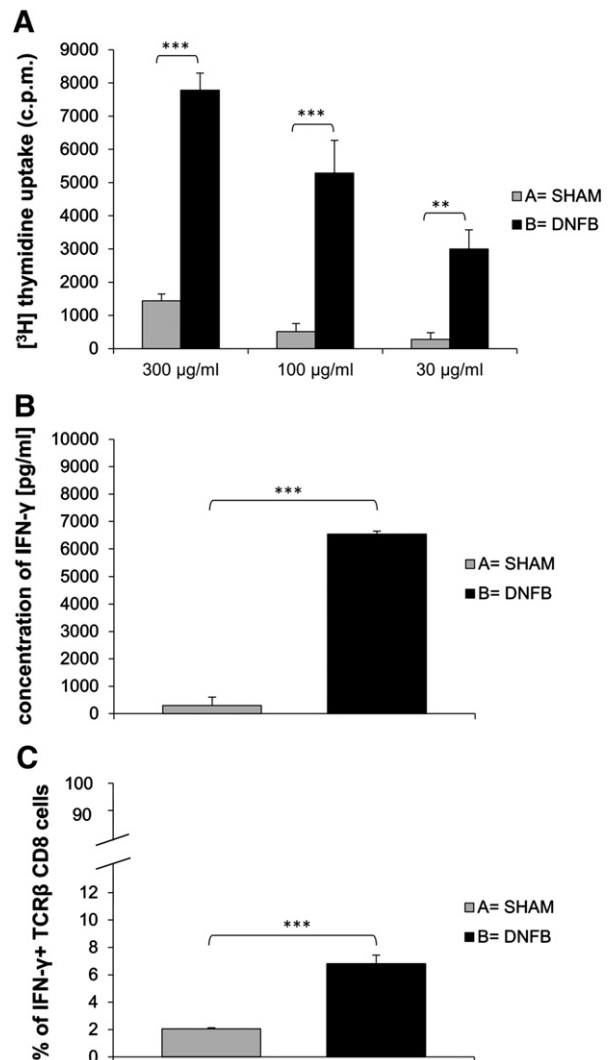


Fig. 2. Evaluation of CHS in vitro. (A) Proliferation of lymph node cells (LNC) in vitro as an alternative read out of CHS. BALB/c mice were contact sensitized by abdominal skin painting with 0.5% DNFB (Group B) or sham sensitized with vehicle alone (Group A). Five days later inguinal and axillary lymph nodes were isolated and 3×10^5 LNC were cultured in 96-U bottom microtiter plates in triplicate in the presence of three fold dilutions of DNP-BSA for 48 h prior to the addition of [^{3} H]-thymidine for an additional 18 h. [^{3} H]-thymidine incorporation was determined by β liquid scintillation counting. Results are presented as mean $\pm$ SE. $n=4$. ** $p \leq 0.01$, *** $p \leq 0.001$. (B) In vitro IFN- γ production as a complementary method to test CHS. BALB/c mice were skin painted with 0.5% DNFB (Group B) or sham immunized (Group A) as described in Material and methods. Five days later axillary and inguinal LN were collected and processed under aseptic conditions and then 3×10^6 LNC were cultured in the presence of 100 μ g/ml DNP-BSA. Cells were distributed in triplicate wells in flat 24 well Falcon plates. After 48 h culture supernatants were collected and then tested for IFN- γ concentration. Results are presented as mean $\pm$ SE. $n=4$. *** $p \leq 0.001$. (C) Evaluation of IFN- γ^+ TCR $\alpha\beta$ CD8 CHS-effector cells by flow cytometry. BALB/c mice were skin painted with 0.5% DNFB (Group B) or sham immunized (Group A) as described in Material and methods. Five days later axillary and inguinal LN were collected and lymph node cells first were stained for surface markers with anti-CD8a-PerCP-Cy5.5 and anti-TCR β -FITC mAbs, and further processed using a fixation buffer and permeabilization buffer. Cells were then stained with anti-IFN- γ -APC. Resultant cells were analyzed by flow cytometry. The percentage of IFN- γ^+ cells was made after gating on CD8 $^+$ TCR β^+ cells. Results are presented as mean $\pm$ SE. $n=4$. *** $p \leq 0.001$.

objective laboratory tests can be used. Inflammatory response in the ears of hapten challenged mice is accompanied with increased vessel permeability that is followed by the movement of fluid containing serum proteins e.g. complement and antibodies to the site of hapten deposition. Observed changes of increased vessel permeability can be easily measured with the use of Evans blue test based on colorimetric method (Askenase et al., 2011; Majewska-Szczepanik et al., 2012).

The swelling of the late 24 hr component of CHS is caused largely by infiltration of leukocytes and by leukocyte-dependent fibrin deposition (Colvin and Dvorak, 1975). These changes can be evaluated by comparison of ear weight of hapten sensitized and challenged vs sham immunized and challenged mice. Then, ear punches used for ear weight estimation can be used for further test. Cell infiltrates in the inflamed ears consist of IFN- γ producing T lymphocytes (Majewska-Szczepanik et al., 2012) and MPO positive neutrophils (Majewska-Szczepanik et al., 2012; Szczepanik et al., 2000). Both parameters can be simply measured in ear tissue homogenates employing ELISA test for IFN- γ detection and colorimetric method to measure MPO activity (Majewska-Szczepanik et al., 2012; Szczepanik et al., 2000). Additionally, data showing IFN- γ in ear tissue extracts were confirmed by IFN- γ production by auricular lymph node cells (ELNC) isolated from DNFB immunized and challenged mice.

Changes observed in the inflamed ear tissue can be also supplemented with in vitro tests showing T cell proliferation and IFN- γ production. This can be easily achieved by culturing skin draining lymph node cells in the presence of hapten-conjugated protein antigen e.g. DNP-BSA (Majewska-Szczepanik et al., 2012). Then culture supernatants can be used for cytokine analysis, whereas lymph node cells pulsed with [^{3} H-thymidine] will allow to measure proliferative response to DNP in vitro. Finally, flow cytometry can be easily used to detect IFN- γ -producing TCR $\alpha\beta$ CD8 CHS-effector cells in axillary and inguinal lymph nodes isolated from DNFB immunized mice.

It should be noted that measurement of IFN- γ concentration in culture supernatants produced by lymph node cells isolated from DNFB sensitized mice that were or were not ear challenged gives us different type of information. Evaluation of IFN- γ secretion by LNC isolated from DNFB immunized but not challenged animals gives us information about IFN- γ production by CHS-effector cells in the place of their induction. On the other hand estimation of IFN- γ secretion by ELNC taken from DNFB immunized and challenged mice gives us knowledge about IFN- γ production by CHS-effector cells at the site of effector response.

In summary, this manuscript shows alternative methods that help to confirm ear swelling measurements with a micrometer, making presented data less subjective and more reliable. However, it should be remembered that alternative methods of CHS evaluation can be used only at one time point whereas measurements with a micrometer can be repeated at different time points which allows the investigation of CHS kinetics.

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