A Novel Model for Human Atopic Dermatitis: Application of Repeated DNCB Patch in BALB/c Mice, in Comparison with NC/Nga Mice

Kyoung-Sun Lee, Eui-Suk Jeong, Seung-Ho Heo, Jin-Hee Seo, Dong-Gu Jeong and Yang-Kyu Choi*

Department of Laboratory Animal Medicine, College of Veterinary Medicine, Konkuk University, Seoul, Korea

The various murine models have contributed to the study of human atopic dermatitis (AD). However, limitations of the models involve low reproducibility and long time to develop AD. In an attempt to overcome these limitations and establish an atopic dermatitis murine model, we repeated the application of 2,4-dinitrochlorobenzene (DNCB) patch in NC/Nga and BALB/c mice, which has advantages in reproduction and cost. For the sensitization, a 1 cm² gauze-attached patch, where 1% or 0.2% DNCB was periodically attached on the back of NC/Nga and BALB/c mice. To estimate how homologous our model was with human atopic dermatitis, clinical, histological and immunological alterations were evaluated. Both strains showed severe atopic dermatitis, increase in subiliac lymph node weight, mast cells, epidermal hyperplasia and serum IgE levels. Though both exhibited a high IL-4/IFN-γ and IL-4/TNF-β ratio in the expression of mRNA, the shifting of DNCB-treated BALB/c mice was increased to more than double that of NC/Nga mice. These results suggest that our DNCB patched model using BALB/c mice were more suitable than NC/Nga mice in demonstrating the immune response. We anticipate that our novel model may be successfully used for pathogenesis of atopic dermatitis and assessment of therapeutic approaches.

Key words: Atopic dermatitis, BALB/c, 2,4-dinitrochlorobenzene (DNCB), NC/Nga

(Received 2 March 2010; Revised version received 15 March 2010; Accepted 17 March 2010)
these models required a relatively longer time for atopic dermatitis induction. Another atopic dermatitis models using various haptens are difficult to paint the haptens on same size in each mouse (Tanguchi et al., 2003; Lee et al., 2006; Sunada et al., 2008). Different sizes cause the different concentration of sensitizer per area and induce the different levels of sensitization of the skin (Friedmann, 2006). Furthermore, intake of the haptens by the mice is not easy to prevent. These limitations weaken the experimental accuracy and data reproducibility. To supplement such weaknesses and develop a more convenient and reproducible model, we used a 2,4-dinitrochlorobenzene (DNCB) and patch in BALB/c and NC/Nga mice. Then, we estimated how homologous our models was with human atopic dermatitis via clinical, histological and immunological alterations.

**Materials and Methods**

**Animals and reagents**

Male BALB/c and NC/Nga mice were purchased from Korea Research Institute of Bioscience and Biotecnology (Daejeon, Korea) and SLC (Shizuoka, Japan) and were 7 weeks old at the initiation of the study. All mice were maintained at the barrier facility of the College of Veterinary Medicine at Konkuk University (Seoul, Korea) and were housed on woodchip bedding (Sani-chip®; Harlan TEKLAD, Madison, WI, USA) with a light-dark cycle of 12:12 h (08:00 to 20:00 h). The room temperature was maintained at 22±2°C with a relative humidity of 50±10%. The animals were fed a sterilized, pelleted diet (2918C®; Harlan TEKLAD, Madison, WI, USA) and had access to autoclaved water through drinking bottles ad libitum. The study was conducted using animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University. 2,4-dinitrochlorobenzene used as a sensitizer was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in acetone-olive oil (BioBasic Inc., Toronto, Ontario, Canada) in a ratio of 4:1. The acetone-olive oil (AOO) was used as vehicle.

**Sensitization and challenge**

The backs of mice were shaved with an electric clipper and depilatory cream, and washed with sterilized PBS-gauzed a day before sensitization. For the sensitization process (Figure 1), a-cm² gauze-attached patch (Tegaderm®; 3M Health Care, MN, USA), applied with 0.1 mL of 1% DNCB in vehicle or vehicle alone was attached to the shaved backs of the animals for 2 day on day 0 and 3. On day 7 and 10, 0.1 mL of 0.2% DNCB in vehicle or vehicle were used to challenge for a day as a previous sensitization.

**Measurement of serum IgE levels**

Mice were anesthetized by intra-peritoneal injection of tribromethanol (125-250 mg/kg, Sigma-Aldrich). Blood samples were obtained from the retro-orbital venous plexus of mice and serum was separated via centrifugation and stored at -80°C until tested. The assessments of IgE were performed using enzyme-linked immunoabsorbent assay kits (BD OptEIA®; BD Pharmingen, San Diego, CA, USA) according to the manufacturer’s protocol. Briefly, the capture antibodies were added to each well in a 96-well ELISA plate (Costar®; Corning Inc., Coming, NY, USA) and incubated to adhere overnight at 4°C. The plates were washed and then blocked with 10% fetal bovine serum in phosphate-buffered saline for 1 h at room temperature (RT). After washing, the diluted standard and samples were added in the plates and incubated for 30 min. After washing, the biotinylated secondary antibodies were added to each well and incubated for 30 min. Plates were washed again and 0.02% streptavidin-alkaline phosphatase was added to each well and incubated for 30 min. The plates were then washed and 0.1 N NaOH was added to each well to stop the enzyme reaction. Absorbance at 405 nm was measured with a spectrophotometer. IgE levels were calculated from the standard curves.

**Histopathological analysis**

Histopathological examinations, skin samples from the back of each mouse were prepared in 10% neutral buffered formalin, embedded in paraffin and cut into four-micrometer-thick sections. The sections were stained with hematoxylin-eosin (HE) to evaluate epidermal hyperplasia and infiltration of immune cells in the dermis, as well as toluidine blue to count the number of mast cells in five random fields (×400) using a computerized image analyzer (MetaMorph 7.5; Molecular Devices, Downingtown, PA, USA).

**Evaluation of skin lesion**

The severity of dermatitis was clinically assessed using previously established methods (Matsuda et al., 1997; Kim et al., 2008). The total scores of skin severity were defined as a sum of the individual score 0, no symptoms; 1, mild; 2, moderate; 3, severe) for each of the following four signs and symptoms: erythema/hemorrhage; edema; excoriation/erosion; dryness. The total dermatitis score was defined as the individual scores indicated above (maximum score: 12).
A mouse model using DNCB patch

Table 1. cytokine-specific primer pair sequences used in RT-PCR

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Oligonucleotide sequences (5'-3')</th>
<th>Size(bp)</th>
<th>Tm(°C)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F: CCC TGA AGT ACG CCA TTG AA R: GGG GTG TTT AAG GTC TCA AA</td>
<td>193</td>
<td>55</td>
<td>20 &amp; 23</td>
</tr>
<tr>
<td>IL-4</td>
<td>F: CGG ATG CGA CAA AAA TCA R: CTT ATC GAT GAA TTC AGC AA</td>
<td>270</td>
<td>60</td>
<td>30 &amp; 35</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: AAC TAG TGG TGC CAG CGG AT R: CTT CAC AGA GCA ATG ACT CC</td>
<td>333</td>
<td>60</td>
<td>25 &amp; 30</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F: AAC GCT ACA CACTGC ATCT TT R: GCT GGA CCT GTG GGT GGT TGT</td>
<td>379</td>
<td>60</td>
<td>32 &amp; 35</td>
</tr>
</tbody>
</table>

2 h at RT. After washing and adding 100 µL of working detector (including detector antibody and avidin-HRP reagent) to each well, the plates were incubated for 1 h at RT and then washed. The substrate solution (0.1 mL) was transferred into each well, incubated in the dark for 30 mins at RT and absorbance was read at 450 and 570 nm. IgE levels in serum were quantified by comparison to the standard.

Semi-quantitative RT-PCR for cytokine-specific mRNA

For detection of cytokine-specific mRNA (IFN-γ, TNF-α and IL-4), total RNA samples were prepared from the skin of DNCB or vehicle-treated mice using the One Step-RNA® reagent (BioBasic Inc., Toronto, Canada) by following the manufacturer’s directions. Aliquots of total RNA (2 mg) were reverse transcribed. A microliter volume of cDNA prepared as described above was used for PCR amplification with PCR premix (Hotstart®; Bioneer Inc., Daejeon, Korea) at two different cycles, unsaturated. Each primer pair sequence and condition was designed on the basis of published gene sequences as indicated in Table 1. The PCR products were electrophoresed on a 1.5 % agarose gel and photographed under UV transillumination. For the quantitative analysis, the mean band intensities in two cycles of each cytokine were normalized to that corresponding β-actin using a densitometer (MultiGauge v3.0 software; Fujifilm Life Science, Tokyo, Japan).

Statistical analysis

All values are presented as means±SD. P<0.05 was considered to be significant in all statistical tests. Data were compared between groups. One-way ANOVA (SPSS 12.0K for Windows, SPSS Institute Inc., USA) was used to compare all pairs of means at the point of same time.

Results

Induction of atopic dermatitis by repeated-application of DNCB

To determine whether atopic dermatitis-like skin lesions were developed by repeated application of DNCB, clinical and histopathological analysis was performed on the back of mice. As shown in Figure 2, the dermatitic lesions of DNCB-treated groups increased significantly, while vehicle-treated groups demonstrated no change. We next performed histopathological analysis using hematoxylin-eosin and toluidine blue stains (Figure 3). The skins of DNCB-treated

Figure 2. (A) Pictures of the clinical observations on the back of mice. (B) Evaluation of clinical dermatitis induced by DNCB and vehicle. The evaluation of clinical dermatitis was based on the areas of gauze which delivered the DNCB and vehicle. Each column shows the mean±SD of 5 mice.

groups distinctly showed epidermal hyperplasia and infiltration of inflammatory cells, including lymphocytes and eosinophils, in the dermis, unlike the vehicle-treated groups. Also, the number of mast cells in DNCB-treated groups was significantly higher than that in vehicle-treated groups (Figure 4). These results indicate that repeated application of DNCB induced epidermal hyperplasia and recruited inflammatory cells to the dermis of the applied site without strain differences between NC/Nga and BALB/c mice.

**Increase in serum IgE by DNCB treatment**

We investigated the changes of serum IgE in our models (Figure 5). Apparent elevation of serum IgE is known as one of the most characteristic features of atopic dermatitis patients. Compared with the vehicle groups, the serum IgE levels of DNCB-treated groups were increased markedly. Though NC/Nga mice showed the higher mean of serum IgE than BALB/c mice, the difference was no statistical significance.

**Figure 3.** Histopathological alterations by DNCB and vehicle. Skins of mice were stained with hematoxylin & eosin (×100, A-D) and toluidine blue (×400, E-H). (A, C, E, G) are vehicle-treated groups and (B, D, F, H) are DNCB-treated groups. ED, epidermis; Dr, dermis; arrow, mast cells. Bar in ×100=200 µm, Bar in ×400=50 µm.

**Figure 4.** Quantification of mast cells in dorsal skin of mice treated with DNCB and vehicle. Skin of mice were stained with toluidine blue and the numbers of mast cell in the dermis of the skin were quantitatively analyzed in 5 high power fields (×400) by a computerized image analyzer. Each column shows the mean±SD of 5 mice.

**Figure 5.** Changes in serum total IgE levels. Blood samples were collected from retro-orbital venous plexus before treatment with DNCB and at 4 days after the last treatment. Serum IgE was quantified using ELISA. Each column shows the mean±SD of 4 mice.
A mouse model using DNCB patch

Increase in local lymph node weight by DNCB treatment

A common method for the identification of skin-sensitizing materials is the murine local lymph node assay, which evaluates the proliferative responses of local lymph node by locally applied materials (Kimber et al., 2001; Kang et al., 2006). Thus, the weight of the local lymph node informs us about a certain magnitude of sensitization by DNCB. Figure 6 shows that the weight of the subiliac lymph node was higher in DNCB-treated NC/Nga and BALB/c mice. This result means DNCB caused proliferation of the lymphocyte in the lymph node.

Changes of cytokine mRNA expression following skin exposure to repeated application of DNCB and vehicle

The Th2-dominant immune response plays a critical role in developing atopic dermatitis. To evaluate the immune response in the skin of our animal models, we studied the expression of IL-4, IFN-γ and TNF-α mRNA (Figure 7).
skins of DNCB-treated groups induced higher IL-4 mRNA expression than that of vehicle-treated groups (Figure 7A). The levels of both Th1 cytokines, IFN-γ and TNF-α, in only DNCB-treated BALB/c mice were decreased apparently while DNCB-treated NC/Nga mice increased (Figure 7B & 7C). There was an increase in the IL-4/IFN-γ and IL-4/TNF-α mRNA ratio (Figure 7D) in both DNCB-treated strains. The increase caused by DNCB treatment was more distinct in BALB/c mice than in NC/Nga mice. These results indicated a more severe shift toward Th2-dominant response after repeated application of DNCB in BALB/c mice than NC/Nga mice.

**Discussion**

Recently, the prevalence of atopic dermatitis has increased two- and threefold around the world, especially in industrialized countries. The onset of atopic dermatitis usually occurs during early infancy and childhood, but it can persist into or start in adulthood (Leung et al., 2004). Also, patients with atopic dermatitis are at a higher risk of developing asthma, food allergy, and allergic rhinitis (Spegele et al., 1998; Gustafsson et al., 2000).

NC/Nga mice have been well-established as a spontaneous animal model for human atopic dermatitis, due to the impaired chromosome 9. But, various limitations, such as low incidence (<50%) and late onset of atopic dermatitis-like lesions, were discovered during atopic dermatitis studies using NC/Nga mice (Shiokara et al., 2004). Although the additional applications of hapten or mite antigen (Kang et al., 2006) on NC/Nga mice is possible, these mice have great disadvantages as an atopic dermatitis model due to long induction time and difficult sensitization results even when using the same concentration of allergens. Another well-known model for atopic dermatitis is the use of transgenic mice overexpressing IL-4, IL-18, IL-31 and Caspase-1, which is useful for pathogenic studies but not for therapeutic studies (Yamakawa et al., 2000; Chan et al., 2001; Tanaka et al., 2001; Dillon et al., 2004). In addition, these mice require a long induction time and commercial exploitation is difficult. Therefore, an inexpensive, reproducible and shortly-inducible model was needed.

In the present study, we established an atopic dermatitis model by the repeated application of a DNCB-patch in BALB/c mice, and compared this model to the NC/Nga mice. The patch is a useful method of applying DNCB to the same location at the same size. Our model was shown to be more cost-effective and more reproducible than previous models because an equivalent concentration of sensitizer per area was successfully applied without any loss, intake and leakage. Clinical and histopathological lesions in human atopic dermatitis show erythema, dryness, epidermal hyperplasia and the pronounced infiltration of lymphocyte and mast cells (Navi et al., 2007; Wollenberg and Klein, 2007). Herein, clinical lesions in both DNCB-treated groups were similar to human atopic dermatitis (Figure 2); histopathological analysis showed the presence of hyperkeratosis, a thick epidermis, and the infiltration of lymphocyte and mast cells demarc (figures 3, 4). Mast cells can be sensitized by the high affinity IgE receptors (FcεRI) on the cell surface and have also been involved in the key effector cells. Mast cells are related to the Th2 response of atopic dermatitis skin. Some reports demonstrated that 66% of mast cells express IL-4 in the lesional skin of atopic dermatitis patients and induce Th2 polarization of skin (Horsmanheimo et al., 1994; Theiner et al., 2006). In the DNCB-treated groups, the increased infiltration of mast cells was similar to that of human atopic dermatitis. The high level of serum IgE helps mast cells sensitize and, in turn, changes into Th2-skewed immune responses. In present study, the elevated level of serum IgE in both DNCB-treated strains was lower than that of other hapten-induced models (Guo et al., 2002; Kim et al., 2008; Sunada et al., 2008). This may be a result of differently applied areas, consumption of the hapten by the animal, exposure period or chemical difference.

In present study, both DNCB-treated strains presented higher IL-4/IFN-γ and IL-4/TNF-α mRNA ratios than the vehicle-treated groups (Figure 7). Many investigators agree that the imbalance of Th1 and Th2 cytokines play an important role in the immunological pathway of atopic dermatitis. T cells, antigen-presenting cells and keratinocytes are regarded as key effector cells in atopic dermatitis (Leung et al., 2004; Akdis et al., 2006). Compared to the normal skin of atopic dermatitis patients, lesional skin exhibits a greater number of Th2 cytokines, IL-4 and IL-5. The former induces the switching signal on B-cell for IgE synthesis and initiates acute skin inflammation in atopic dermatitis, while the latter engages in eosinophil development and survival and maintains chronic inflammation (Hamid et al., 1994; Akdis et al., 2006). In addition, they inhibit the differentiation of naïve T-cells toward Th1 and the production of human beta defensin (HBD)-3, an antimicrobial peptide induced by TNF-α and IFN-γ (Opal and DePal, 2000; Nomura et al., 2003). Therefore, the increased IL-4/IFN-γ and IL-4/TNF-α mRNA ratios by DNCB treatment demonstrated the induction of Th2-dominant response and was associated with the high level of serum IgE (Figure 5).

In the mRNA expression of vehicle-treated groups, an interesting finding was the apparent increase in IL-4 mRNA.
levels of BALB/c mice in comparison to that of NC/Nga mice (Figure 7A). However, the IL-4/IL-10 and IL-4/TNF-α mRNA ratios showed no difference between NC/Nga and BALB/c mice (Figure 7D), because the Th1 cytokines were increased relatively (Figures 7B & 7C). This indicated that the immune response of BALB/c mice was biased toward Th2, which was compensated for by the relative increase of Th1 cytokine. Consequently, this homeostasis keeps the balance of the immune response in vehicle-treated groups. However, the repeated applications of DNCB broke the balance of Th1 and Th2, and prompted a shift toward the Th2-dominant response. Comparing with the shifting of DNCB-treated NC/Nga mice, BALB/c mice were increased by more than double. These results indicated the Th2-skewed immune responses by DNCB were clearly different depending on strains.

In conclusion, using DNCB-patches in BALB/c and NC/Nga mice, we applied same concentration of DNCB on same area. Though both DNCB-treated strains showed typical atopic dermatitis lesion, BALB/c mice was more suitable than NC/Nga mice in demonstrating the immune response. This method in BALB/c mice appeared to be more convenient and reproducible than the previous models, and shortened the time taken to induce atopic dermatitis. Considering the cost and tendency toward Th2-skewed immune response, the BALB/c model is more advantageous than the NC/Nga model. Our model will be helpful for both atopic dermatitis pathogenesis and assessment of therapeutic approaches.

Acknowledgments

This work was supported by the Brain Korea 21 project funded by Korea Research Foundation.

References


