An immunohistochemical study of the nasal mucosa after trichloroacetic acid treatment for allergic rhinitis

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Background: Trichloroacetic acid (TCA) treatment of allergic rhinitis proved clinically satisfactory. In the present study, for the purpose of providing evidence to support the effectiveness of the treatment, we attempted to compare the number of infiltrating Th2 and Th1 cells in specimens obtained from the TCA-treated inferior turbinate mucosae group with the non-treated group.

Methods: Specimens were obtained from 21 patients with house dust mite allergic rhinitis who underwent turbinectomy combined with surgery for a deviated septum after unilateral TCA application for relief from persistent nasal symptoms. In each specimen, Th2 and Th1 cells were identified using immunohistochemical staining, and their numbers were counted to obtain each cell count per unit specimen area (/mm²).

Results: In TCA-treated mucosae, the mean cell count of Th2 cells was 4.93 ± 2.83 cells/mm², while that in non-treated mucosae was 40.87 ± 15.63. The mean Th1 cell count in TCA-treated mucosae was 45.34 ± 25.34 cells/mm², while it was 124.32 ± 54.91 cells/mm² in non-treated mucosae. There was a statistically significant difference in the cell count between TCA-treated and non-treated specimens for both cell types.

Conclusions: TCA application inhibited Th2 cell infiltration, implying that chemosurgery with TCA for allergic rhinitis treatment was clinically effective with good prognosis.

Key words: Th2, Th1, allergic rhinitis, trichloroacetic acid

Introduction

We have reported elsewhere the therapeutic effectiveness of trichloroacetic acid (TCA) application for the treatment of allergic rhinitis (AR),1,2 following its first introduction in 1986. Since that time, we have applied this method in approximately 5,000 cases with AR with satisfactory clinical results. In our method, 80 w/v% TCA is applied using cotton applicators to the inferior turbinates of the patient bilaterally under appropriate topical anesthesia as a one-day procedure. According to our clinical statistics,3 symptomatic improvement was found in 72.5% cases for nasal obstruction, approximately 60% for sneezing, and 50% for watery nasal discharge.

The clinical use of TCA the hypertrophic inferior turbinates actually began in the early twentieth century for nasal obstruction due to simple hypertrophic rhinitis and proved to be effective without showing any side effects.3-7 We followed the same method for the treatment of AR, based on the assumption that TCA application should be effective in controlling local allergic reactions in the nasal mucosae of the inferior turbinates of AR patients.

To prove the validity of our assumption, we have made several histological as well as immunohistological studies until the present time. Among them, Yao et al. compared the specimens obtained from the TCA-treated and non-treated sides of the inferior turbinate of the same patient who underwent turbinectomy a certain period after the TCA application, and found that the numbers of both infiltrating activated eosinophils8 and infiltrating mast cells positive for tryptase were significantly lower in the TCA-treated mucosae than in the non-treated mucosae.9 However, these results appeared to be only indirect evidence of the local
suppression of allergic reaction following TCA application.

More recently, it has been reported that the CD8 and CD4 T cells have the major function of T cells in the immune system. In particular, CD4 T cells work as helper T cells to activate and gather other cells at local sites by discharging different types of cytokines. Further, CD4 T cells were reported to move to the lymphatic follicles and activate B cells, or diverge into the tissues as Th1 cells, Th2 cells, and Th17 cells, which are the differentiated subsets of CD4 T cells, where they start to control other cells. Among the three types, Th1 cells have the function of gathering and activating phagocytic cells, while Th2 cells gather eosinophils, basophils, and mast cells, and protect them from epithelial disorders.

It has also been reported that alteration of the chromatin structure occurs during the differentiation of CD4 T cells into their subsets. For the alteration, different types of transcription modulating factors work together. Among them, T-bet is specifically for guiding the differentiation of Th1 cells, while GATA-3 is specific for Th2 cell differentiation. With the progression of the differentiation, one of the transcription modulating factors becomes dominant, and the differentiation into one of these cell types proceeds. This process of progression is known as polarization. It has been assumed that the mechanism by which AR develops depends heavily on the pathological condition in which the polarization of predominant Th2 cell differentiation occurs at the local sites of the nasal mucosae. I.e., Th2 cells are considered to produce the IgE antibody when they receive antigenic stimulation at the local site, and then allergic tissue reactions are caused by the action of chemical mediators at the local sites.

Thus, if possible, it would be desirable to directly investigate the distribution of Th2 cells in the nasal mucosae after TCA application in order to prove the effect of TCA for the local suppression of allergic reactions in the nasal mucosae. Until relatively recently, however, only the indirect method has been attempted by the authors to evaluate the degree of Th2 cell infiltration at local sites. In 2001, Yao et al. confirmed the expression of thymus and activation-regulated chemokine (TARC), which was known to induce the expression of thymus and activation-regulated chemokine (TARC), 19,20 which were known to induce the expression of Th2 cell differentiation. 15  With the progression of the differentiation factors becomes dominant, and the differentiation into one of these cell types proceeds. This process of progression is known as polarization. 15  It has been postulated that Th2 cell infiltration would be inhibited beneath the epithelial layer and, as a result, type I allergic reactions would be suppressed by the use of TCA, further research to obtain more direct evidence proving the local suppression of Th2 cell infiltration after TCA application was warranted.

Therefore, in the present study, we introduced the immunohistochemical method for identifying the number of infiltrating Th2 cells in the specimen of the nasal mucosae obtained from the TCA treated and non-treated sides of the inferior turbinates of patients with AR. The number of Th1 cells in the same specimen was also assessed as a reference, because T-cell subsets may undergo differentiation through their interaction. In the present study, our objective was to obtain direct evidence of the suppression of local allergic reactions in the nasal mucosae after TCA treatment.

Materials and Methods

Background of the subjects and specimens

From June 2005 to April 2007, a total of 205 patients underwent TCA treatment for allergic rhinitis after we obtained their informed consent at our clinic. Thirty-eight of the 205 patients had TCA treatment on only one side of the nasal cavity because of anatomic difficulties, mainly due to a severely deviated nasal septum. Of the 38 patients, 31 cases were diagnosed as house dust mite allergic rhinitis based on the results of clinical examination, a provocation test, clinical symptoms, and an eosinophil count in the nasal discharge. At the time of obtaining informed consent, we clearly explained that the effect of TCA treatment could be less remarkable if there was anatomic difficulty inhibiting bilateral application of TCA. Even so, the 38 cases willingly agreed to undergo unilateral TCA application.

As we had suspected, among the 31 cases with house dust mite allergic rhinitis, 21 cases continued to have nasal symptoms, nasal obstruction in particular, even after the TCA treatment. For complete relief from their symptoms, we then performed reconstructive surgery for deviated nasal septum combined with bilateral inferior turbinectomy, after again obtaining the patients' informed consent. The patients consisted of 11 men and 10 women with an average age of 33 ± 19 years. The average period from the TCA treatment to the subsequent surgery
An immunohistochemical study of the nasal mucosa after trichloroacetic acid treatment for allergic rhinitis

was 122 ± 41 days.

Thus, we were able to obtain 21 sets of specimens of turbinate mucosae from both the TCA-treated (non-deviated) and non-treated (deviated) sides from the 21 cases. An immunohistochemical study was then conducted to compare the specimens obtained from the TCA-treated and non-treated sides in each of the 21 pairs.

Preparation of specimens
Immediately after the surgery, each of the bilateral turbinate specimens was preserved in a 10% formalin at room temperature. We then performed an immunohistochemical study of the specimens in the form of 3 μm serial sections. The first section was stained with hematoxylin and eosin. For the subsequent sections, we performed immunohistochemical staining, in which we used the anti-human/rabbit CKR4 antibody (Santa Cruz Bio., Inc., No. sc-7936, CA, USA, dilution 1:100, polymer reagent [for rabbits, DakoCytomation/EnVision + Dual Link No. K4063], DAB coloring, hematoxylin nuclear staining), and anti-human/mouse CCR5 antibody (Genzyme-Techne, Minneapolis, lot AJB06, MN, USA, dilution 1:100, polymer reagent [for mice, DakoCytomation/EnVision + Dual Link No. K4063], DAB coloring, hematoxylin nuclear staining). As the negative control, we used DakoCytomation N-Universal Negative Control (Rabbit) Co. N1699, Glostrup, Denmark, for the former, and DakoCytomation N-Universal Negative Control (Mouse) Co. N1698, Glostrup, Denmark, for the latter.

Method of measurement of cell counts
We observed the distribution of Th2 and Th1 cells, and counted the number of each cell type in the specimens obtained from both the TCA-treated and non-treated sides based on the identification method described below. The observation and counting of Th2 cells were made at two different layers of the lamina propria of the turbinate mucosae, i.e., the superficial layer up to a depth of 120 μm beneath the surface epithelium, and the deeper layer. The superficial layer corresponds to the range within which the TCA application was reported to be effective.1,2,18,22 In each specimen, we identified Th2 cells in anti-CKR4 antibody-positive cells when we discovered cells with brownish and partially granular stained cytoplasm and an apparent nucleus. Th1 cells were identified in anti-CCR5 antibody-positive cells if we discovered cells with cytoplasm of a similar feature.

![Figure 1](image1.png)

Figure 1. A view of the specimen obtained from the non-TCA treated side of a 28-year old female Immunohistochemical staining of the superficial layer using the anti-human/rabbit CKR4 antibody (10× magnification)
Typically stained Th2 cells are shown in rectangular frames "a" and "b" and are magnified in Figures 2 and 3, respectively.

![Figure 2](image2.png)

Figure 2. A magnified view of the rectangular frame "a" in Figure 1 (40×5 magnification)
Two positive cells with cytoplasm containing brown-stained granules that were identified as Th2 cells are indicated by arrows.

![Figure 3](image3.png)

Figure 3. A magnified view of the rectangular frame "b" in Figure 1 (40×5 magnification)
Three cells similar to those shown in Figure 2 are indicated by arrows.
**Figure 4.** A view of the specimen obtained from the TCA-treated side of the same subject shown in Figure 1 on the 114th day after TCA treatment. Immunohistochemical staining of the superficial layer using the anti-human/rabbit CKR4 antibody (10 × 5 magnification). Because of the treatment, the tissue was only partially lined by stratified squamous epithelium, and the volume of gland tissue had decreased.

**Figure 5.** A magnified view of the rectangular frame shown in Figure 4 (40 × 5 magnification). The arrow indicates a Th2 cell.

**Figure 6.** A view of the specimen obtained from the non-TCA treated side of a 28-year old female. Immunohistochemical staining of the layer between the epithelium and the glandular layer using the anti-human/rabbit CCR5 antibody (10 × 5 magnification). Typically stained Th1 cells were identified. The distribution was found throughout the lamina propria mucosae.

**Figure 7.** A magnified view of the rectangular frame shown in Figure 6 (40 × 5 magnification). Six Th1 cells are indicated by arrows.

**Figure 8.** A view of the deeper layer of the same specimen as shown in Figure 6 (10 × 5 magnification). Th1 cells are clearly seen.

**Figure 9.** A magnified view of the rectangular frame shown in Figure 8 (40 × 5 magnification). Seven Th1 cells are indicated by arrows.
An immunohistochemical study of the nasal mucosa after trichloroacetic acid treatment for allergic rhinitis

Figure 10. A view of the specimen obtained from the TCA-treated side of a 28-year old female
Immunohistochemical staining of the layer between the epithelium and the glandular layer using the anti-human/rabbit CCR 5 antibody (10 × 5 magnification)
A few Th1 cells are clearly seen.

Figure 11. A magnified view of the rectangular frame shown in Figure 10 (40 × 5 magnification)
Four Th1 cells are indicated by arrows.

Figure 12. A view of the deeper layer of the same specimen shown in Figure 10 (10 × 5 magnification)
Th1 cells are clearly seen.

Table 1. Comparison of the cell counts of Th2 and Th1 cells in the specimens of TCA-treated vs. non-treated (numbers/mm²)

<table>
<thead>
<tr>
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<th>TCA-treated (numbers/mm²)</th>
<th>Non-treated (numbers/mm²)</th>
</tr>
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<tbody>
<tr>
<td>Th2 cells**</td>
<td>4.93 ± 2.83</td>
<td>40.87 ± 15.63</td>
</tr>
<tr>
<td>Th1 cells**</td>
<td>45.34 ± 25.34</td>
<td>124.32 ± 54.91</td>
</tr>
</tbody>
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**P < 0.01

Figure 13. A magnified view of the rectangular frame shown in Figure 12 (40 × 5 magnification)
Four Th1 cells are indicated by arrows.
Figures 1–13 show typical examples obtained from a 28-year-old female. To count the number of Th2 cells, the cell count in the entire area at each layer of each specimen was obtained at 20 × 10 magnification. For Th1 cells, on the other hand, we arbitrarily selected three spots from the three different layers of each specimen, i.e., the subepithelial layer, the deeper intravenous layer and the intermediate layer between the two, and the number of Th1 cells was counted in each spot by attaching a micrometer (1 cm²) to the eye lens at 20 × 10 magnification. The mean value was the obtained. We then measured the area of each specimen with a Digital Planimeter (Koizumi Sokki Mfg. Co., Ltd., Nagaoka, Niigata), and the numbers of infiltrating Th2 cells and Th1 cells per mm² of the specimen were finally determined.

Statistical methods
Two-way analyses of variance (ANOVA) were performed using StatMate III software on the obtained data of cell counts of each cell type, with the conditions of TCA application and non-application as variables. The level of significance is shown in Table 1.

Ethical considerations
In addition to obtaining informed consent prior to each clinical procedure, permission was obtained from each subject to use the obtained specimens for research purposes. The study was conducted in accordance with the guidelines of the Ethical Committee of the Kitasato University School of Medicine.

Results
Distribution patterns of the Th2 and Th1 cells
Distribution in specimens obtained from the non-treated side revealed that there were no Th2 cells in the covering epithelium in any of the specimens. In the lamina propria mucosae, Th2 cells were found only in the superficial layer just beneath the surface epithelium up to a depth of nearly 120 μm beneath the surface epithelium (Figures 1–3). In that layer, they were found mainly in the areas surrounding the acini and the conduits of the glands. Th1 cells were found almost evenly throughout the specimens from the area just beneath the surface epithelium to the cavernous plexus layer (Figures 6–9). Distribution in the specimens obtained from the TCA-treated side revealed that the pattern of distribution of both Th2 cells (Figures 4, 5) and Th1 cells (Figures 10–13) in the specimens obtained from the TCA-treated side was essentially similar to those described above.

Number of Th2 and Th1 cells per unit area (/mm²) in TCA-treated and non-treated specimens as shown in Table 1 compares the number of Th2 and Th1 cells observed in the specimens obtained from the turbinate mucosae between the TCA-treated and non-treated sides. As shown in the table, the mean cell counts of Th2 and Th1 cells in the TCA-treated side were 4.93 ± 2.83 and 45.34 ± 25.34, respectively, while the counts in the non-treated side were 40.87 ± 15.63 and 124.32 ± 54.91, respectively. In both Th2 and Th1 cells, the number of cells was significantly less in the TCA-treated side than that in the non-treated side.

All of the controls that rule out non-specific reactions, which we performed at the same time with staining to confirm the identification of Th2 and Th1 cells, were confirmed to be negative.

Discussion
In the present study, we discovered that the number of infiltrating Th2 and Th1 cells was significantly smaller in the TCA-treated inferior turbinate mucosae. The fact that the number of Th2 cells decreased after TCA treatment proved the effectiveness of TCA treatment for local suppression of allergic reactions. Furthermore, these results should support the validity of our previous report on the inhibition effect of TCA for activated eosinophils and infiltrating mast cells positive for tryptase alone.

The number of infiltrating Th2 cells in the non-treated mucosae was also low contrary to pre-experiment expectations. The reasons for these observations may be as follows. There was a 2- or 3-day period of preoperative hospitalization prior to the operation, during which the patients would not have been exposed to the antigen. For ethical reasons, anti-allergic medication was not discontinued prior to the operation. This medication may have an influence on the number of Th2 cell infiltrations. In addition, it is possible that treatment of one side of the nasal cavity may have exerted an inhibitory effect on the allergic reactions on the other side by a reflex mechanism, considering that even treatment on only one side yielded an overall improvement in symptoms in some patients. The reflex in accordance with TCA treatment on one side may have influenced these results. Furthermore, the fact that the chemokine receptors that are peculiarly expressed on the Th2 cells vary in terms of their functions and time-points of expression should also be considered. In any event, the aforementioned results are a comparison of the inferior turbinate mucosae from the same individual; and it is therefore natural to consider that both external antigenic stimulation and intrinsic allergic reaction stimulation
influence the TCA-treated and non-treated inferior turbinate mucosae equally, and that TCA treatment factors did in fact produce a significant difference between infiltrating Th2 and Th1 cells.

Hence, the issue here is the possibility that the increase in the number of infiltrating cells could be caused by increased nasal resistance in nasal respiration, including a deviated nasal septum. Even though the comparison of the cell counts in the present study was made for specimens obtained from patients with an asymmetrical nasal airway caused by a deviated nasal septum, the effect of the deviation can be disregarded because of the results in our previous study,27 which compared mucosae in the deviated and non-deviated sides without TCA treatment, revealing that the deviation factor had no significant effect on Th2 or Th1 cell distribution. Therefore, the deviation factor effect need not be considered in the present study.

The definite function of Th1 cells has not yet been fully explored, although it is possible that they play an important role in cellular immune competence.28,29 Judging from the results of the present study, revealing a decrease in the number of Th1 cells as an effect of TCA application, the procedure may provoke an adverse effect on the cell immunity system in the nasal mucosa. Since our first application of TCA to allergic nasal mucosa, however, we have found no notable clinical signs or symptoms apparently caused by local suppression of the cellular immune mechanism.

The local application of TCA to allergic nasal mucosa is a useful clinical approach for any otolaryngologist with knowledge of the anatomy of the nasal cavity. The procedure can be performed safely and easily as a one-day procedure on an outpatient basis. This procedure proved to be a treatment modality that inhibits type I allergic reactions, the pathogenic mechanism underlying the development of allergic rhinitis, and improves patient quality of life.

An immunohistochemical study comparing the distribution of Th2 cells between the specimens obtained from TCA-treated and non-treated inferior turbinate mucosae revealed that the number of infiltrating Th2 cells was significantly smaller in the TCA-treated side. This result provides objective evidence supporting the usefulness of TCA treatment for local suppression of type I allergic reactions, the pathogenic mechanism underlying the development of allergic rhinitis. This method is applicable in a one-day procedure on an outpatient basis and proved to be useful for the treatment of allergic rhinitis.

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